



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 47/48, C07K 14/65		A1	(II) International Publication Number: WO 95/02421 (43) International Publication Date: 26 January 1995 (26.01.95)
(21) International Application Number: PCT/US94/08000		Cambridge, MA 02139 (US). MCGRATH, John, P. [US/US]; 14 Fort Washington Place, Cambridge, MA 02139 (US).	
(22) International Filing Date: 18 July 1994 (18.07.94)		(74) Agents: BROOK, David, E. et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).	
(30) Priority Data: 08/094,534 16 July 1993 (16.07.93) US		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).	
(60) Parent Application or Grant (63) Related by Continuation US Filed on 08/094,534 (CIP) 16 July 1993 (16.07.93)		Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
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(54) Title: TRANSPERRIN RECEPTOR SPECIFIC LIGAND-NEUROPHARMACEUTICAL AGENT FUSION PROTEINS			
(57) Abstract			
<p>The present invention pertains to a method for delivering a neuropharmaceutical agent across the blood brain barrier to the brain of a host. The method comprises administering to the host a therapeutically effective amount of a ligand-neuropharmaceutical agent fusion protein wherein the ligand is reactive with a brain capillary endothelial cell receptor. Other aspects of this invention include a delivery system comprising a ligand reactive with a brain capillary endothelial cell receptor which has formed a fusion protein with a neuropharmaceutical agent. The fusion proteins are also aspects of this invention.</p>			

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TRANSFERRIN RECEPTOR SPECIFIC LIGAND-NEUROPHARMACEUTICAL  
AGENT FUSION PROTEINS

Description

Background

5        The capillaries that supply blood to the tissues of the brain constitute the blood brain barrier (Goldstein *et al.* (1986) Scientific American 255:74-83; Pardridge, W.M. (1986) Endocrin. Rev. 7:314-330). The endothelial cells which form the brain capillaries are different from 10 those found in other tissues in the body. Brain capillary endothelial cells are joined together by tight inter-cellular junctions which form a continuous wall against the passive movement of substances from the blood to the brain. These cells are also different in that they have few 15 pinocytic vesicles which in other tissues allow somewhat unselective transport across the capillary wall. Also lacking are continuous gaps or channels running through the cells which would allow unrestricted passage.

20       The blood-brain barrier functions to ensure that the environment of the brain is constantly controlled. The levels of various substances in the blood, such as hormones, amino acids and ions, undergo frequent small fluctuations which can be brought about by activities such as eating and exercise (Goldstein *et al.*, cited supra). If 25 the brain were not protected by the blood brain barrier from these variations in serum composition, the result could be uncontrolled neural activity.

30       The isolation of the brain from the bloodstream is not complete. If this were the case, the brain would be unable to function properly due to a lack of nutrients and because of the need to exchange chemicals with the rest of the

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body. The presence of specific transport systems within the capillary endothelial cells assures that the brain receives, in a controlled manner, all of the compounds required for normal growth and function. In many 5 instances, these transport systems consist of membrane-associated receptors which, upon binding of their respective ligand, are internalized by the cell (Pardridge, W.M., cited supra). Vesicles containing the receptor-ligand complex then migrate to the abluminal 10 surface of the endothelial cell where the ligand is released.

The problem posed by the blood-brain barrier is that, in the process of protecting the brain, it excludes many potentially useful therapeutic agents. Presently, only 15 substances which are sufficiently lipophilic can penetrate the blood-brain barrier (Goldstein *et al.*, cited supra; Pardridge, W.M., cited supra). Some drugs can be modified to make them more lipophilic and thereby increase their ability to cross the blood brain barrier. However, each 20 modification has to be tested individually on each drug and the modification can alter the activity of the drug. The modification can also have a very general effect in that it will increase the ability of the compound to cross all cellular membranes, not only those of brain capillary 25 endothelial cells.

Summary of the Invention

The present invention pertains to a method for delivering a neuropharmaceutical agent across the blood brain barrier to the brain of a host. The method comprises 30 administering to the host a ligand-neuropharmaceutical agent fusion protein wherein the ligand is reactive with a brain capillary endothelial cell receptor. The ligand of the fusion protein is an intact ligand to a brain capillary endothelial cell receptor or a receptor-binding fragment

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thereof. Alternatively, the ligand can be an antibody or immunoreactive fragment thereof that is reactive with a brain capillary endothelial cell receptor. The neuropharmaceutical agent of the fusion protein is a 5 protein, polypeptide or peptide. The fusion protein is administered under conditions whereby binding of the ligand to a receptor on a brain capillary endothelial cell occurs and the neuropharmaceutical agent is transferred across the blood brain barrier in a pharmaceutically active form and 10 in a therapeutically effective amount.

The present invention also pertains to a delivery system comprising a ligand-neuropharmaceutical agent fusion protein wherein the ligand is reactive with a brain capillary endothelial cell receptor. This delivery system 15 transports the neuropharmaceutical agent across the blood brain barrier in a pharmaceutically active form when the fusion protein is administered in vivo. The present invention also pertains to the fusion proteins themselves which have both ligand binding and neuropharmaceutical 20 characteristics.

Fusion proteins which include a brain capillary endothelial cell receptor ligand and an antibody, or immunoreactive fragment thereof, that is itself reactive with a brain capillary endothelial cell receptor are other 25 aspects of the present invention. In these aspects, neuropharmaceutical agents can be conjugated to the fusion proteins by cleavable or noncleavable linkers for transport of these agents across the blood brain barrier. Also pertaining to the present invention are a delivery system 30 incorporating these fusion proteins and a method for delivering the neuropharmaceutical agent across the blood brain barrier in a pharmaceutically active form by administering to the host a fusion protein-neuropharmaceutical agent conjugate in a therapeutically 35 effective amount.

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Presently available means for delivering neuropharmaceutical agents to the brain are limited in that they are invasive. The delivery system of the present invention is non-invasive and can utilize readily available 5 ligands reactive with brain capillary endothelial cell receptors as carriers for neuropharmaceutical agents. The delivery system is advantageous in that the ligands, when formed as part of a fusion protein with neuropharmaceutical agents, are capable of transporting the neuropharmaceutical 10 agents across the blood brain barrier without being susceptible to premature release of the neuropharmaceutical agent prior to reaching the brain side of the blood brain barrier. The delivery system is similarly advantageous when the neuropharmaceutical agent is conjugated to the 15 fusion protein by a noncleavable bond.

Description of the Drawings

Figure 1 is a graphic representation of rat brain uptake of  $^{14}\text{C}$ -labelled murine monoclonal antibody (OX-26) to rat transferrin receptor in rats where the percent 20 injected dose of radiolabelled antibody per brain and per 55  $\mu\text{l}$  of blood is plotted versus time post-injection.

Figure 2 is a histogram illustrating time dependent changes in the disposition of radiolabelled OX-26 between brain parenchyma and vasculature.

25 Figure 3 is a histogram illustrating the biodistribution of antibody 128.1 and control IgG in a cynomolgus monkey.

Figure 4 is a restriction enzyme map of bacterial 30 plasmid pAT3442 which additionally shows the human IgG3 and transferrin gene regions.

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Figure 5A is a restriction enzyme map of the CH1-hinge-transferrin region of clone pATX.

Figure 5B is a restriction enzyme map of the CH1-hinge-transferrin region of clone pATXX.

5 Figure 5C is a restriction enzyme map of the pre-pro NGF-hinge-transferrin region of clone pATXXNGF.

Figure 6 is a restriction enzyme map of clones pUCNCF1 and pUCNCF2 which contain the NGF gene in opposite orientations.

10 Figure 7 is a set of restriction enzyme maps that depict the formation of plasmid pcDNA1/AmpNHT with fragments from clones pUCNCF2 and pATXXNGF.

Figure 8 is a restriction enzyme map of clone CD51neg1.

15 Figure 9A-9B is the DNA sequence between the XhoI and EagI sites of clone CD51neg1, where the coding sequences for the CD5 Leader and IgG1 Exons 1, 2 and 3 are displayed in larger, bold characters (Seq.I.D.NO.8).

20 Figure 10 is a graphic representation of the competition between either recombinant human transferrin or NHT fusion protein and radioactively labeled transferrin when binding to human placental transferrin receptors.

Figure 11A is a graphic representation of the inducing effect of NGF on the sprouting of neurites from PC12 cells.

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Figure 11B is a graphic representation of the inducing effect of NHT fusion protein on the sprouting of neurites from PC12 cells.

Figure 12A is a restriction enzyme map of plasmids D1  
5 and d1.

Figure 12B is a restriction enzyme map of plasmids C4 and C\*.

Figure 12C is a restriction enzyme map of plasmid C4-NHT.

10 Figure 12D is a restriction enzyme map of plasmid g\*.

Figure 12E is a restriction enzyme map of plasmid H45.  
Figure 12F is a restriction enzyme map of plasmid gH.

Detailed Description

The method for delivering a neuropharmaceutical agent across the blood brain barrier to the brain of a host 15 comprises administering to the host a ligand-neuropharmaceutical agent fusion protein wherein the ligand is reactive with a receptor present on a brain capillary endothelial cell. The method is conducted under conditions whereby the ligand binds to the receptor on the brain 20 capillary endothelial cell and the neuropharmaceutical agent is transferred across the blood brain barrier in a pharmaceutically active form and in a therapeutically effective amount.

25 The ligand-neuropharmaceutical agent fusion protein, which has both ligand binding and neuropharmaceutical characteristics, can be produced as a contiguous protein by using genetic engineering techniques. Gene constructs can be prepared comprising DNA encoding the ligand fused to DNA

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encoding the protein, polypeptide or peptide to be delivered across the blood brain barrier. The ligand coding sequence and the agent coding sequence are inserted in the expression vectors in a suitable manner for proper expression of the desired fusion protein. The gene fusion is expressed as a contiguous protein molecule containing both a ligand portion and a neuropharmaceutical agent portion. For example, sequences encoding neurotrophic agents such as NGF (nerve growth factor) or CNTF (ciliary neurotrophic factor) can be fused with the sequence 5 encoding transferrin to create chimeric polypeptides that will be expressed and subsequently transported across the 10 BBB via the transferrin receptor.

The genetic engineering techniques are often used to 15 insert linker DNA sequences between the ligand and the neuropharmaceutical agent DNA encoding sequences. These linker DNA sequences can be expressed as part of the fusion protein. For example, specific segments of the constant region of an antibody, including the hinge region, can be 20 inserted between the ligand and the neuropharmaceutical agent. These expressed insertions serve to separate the ligand from the neuropharmaceutical agent and may facilitate the proper folding of the expressed ligand or agent into its proper conformation. When the insertions 25 are segments from the constant region of antibodies that are syngeneic to the host, they have the added advantage of having reduced immunogenicity when administered.

The host can be an animal susceptible to a 30 neurological disorder (i.e., an animal having a brain). Examples of hosts include mammals such as humans, domestic animals (e.g., dog, cat, cow or horse), mice and rats.

The neuropharmaceutical agent can be an agent having a therapeutic or prophylactic effect on a neurological disorder or any condition which affects biological 35 functioning of the central nervous system. Examples of

neurological disorders include cancer (e.g. brain tumors),  
Acquired Immune Deficiency Syndrome (AIDS), stroke,  
epilepsy, Parkinson's disease, autoimmune diseases such as  
multiple sclerosis, neurodegenerative disease, trauma,  
5 depression, Alzheimer's disease, migraine, pain, or a  
seizure disorder. Classes of neuropharmaceutical agents  
which can be used in this invention include proteins and  
polypeptides used to treat or prevent a neurological  
disorder. Examples of proteins include growth factors  
10 (e.g. nerve growth factor (NGF)), ciliary neurotrophic  
factor (CNTF), brain-derived neurotrophic factor (BDNF),  
glial cell-line derived neurotrophic factor (GDNF),  
neurotrophins 3,4 and 5 (NT-3,4 and 5) or fibroblast growth  
factor (FGF), lymphokines or cytokines (e.g. interferon or  
15 interleukins (IL-2)) or antagonists thereof, CD4 and  
superoxide dismutase (including soluble portions thereof),  
dopamine decarboxylase and tricosanthin. Examples of  
polypeptides include somatostatin analogues and  
enkephalinase inhibitors.

20 The ligand of the fusion protein is any polypeptide or  
protein that is capable of binding with specificity to a  
receptor on brain capillary endothelial cells. These  
receptors are normally located on the luminal surfaces of  
these endothelial cells when they line the inner portion of  
25 the brain blood vessels. A particularly preferred ligand  
family is transferrin and any transferrin derivatives which  
retain transferrin receptor-binding activity.

Serum transferrin is a monomeric glycoprotein with a  
molecular weight of 80,000 daltons that binds iron in the  
30 circulation and transports it to the various tissues (Aisen  
et al. (1980) *Ann. Rev. Biochem.* **49**: 357-393; MacGillivray  
et al. (1981) *J. Biol. Chem.* **258**: 3543-3553). The uptake  
of iron by individual cells is mediated by the transferrin  
receptor, an integral membrane glycoprotein consisting of  
35 two identical 95,000 dalton subunits that are linked by a

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disulfide bond. The number of receptors on the surface of a cell appears to correlate with cellular proliferation, with the highest number being on actively growing cells and the lowest being on resting and terminally differentiated 5 cells. Jeffries *et al.* (*Nature* 312, pp. 167-168 (November 1984)) used monoclonal antibodies to show that brain capillary endothelial cells have a high density of transferrin receptors on their cell surface.

10 Fusion proteins comprising ligands and neuropharmaceutical agents can also be prepared where the ligands are reactive with other receptors, besides the transferrin receptor, which can also mediate the 15 endocytotic or transcytotic process of transporting macromolecules across the blood-brain barrier. These 20 receptors are also on the cell surface of the endothelial cells which line brain vessels.

Among the receptor types are those that react with insulin-like growth factors 1 or 2 (IGF 1 or 2) or insulin 25 and derivatives of these ligands which retain receptor-binding activity. The therapeutic agents which can be conjugated to the ligands include the above-mentioned proteins such as nerve growth factor, ciliary neurotrophic factor, brain-derived neurotrophic factor, superoxide dismutase, CD-4 or anti-amyloid antibody.

The term receptor is intended to encompass the entire receptor or ligand-binding portions thereof. These portions of the receptor particularly include those regions sufficient for specific binding of the ligand to occur.

30 Ligands which can bind with specificity to brain capillary endothelial cell receptors include antibodies or antibody fragments that can bind with these receptors. These antibodies or antibody fragments are as capable of binding to the brain capillary endothelial cell receptors 35 as the nominal receptor ligands. Upon binding of the

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antibodies to the receptors, transferal of the antibody and any attached agent across the blood brain barrier occurs. The agent can be attached by any acceptable means for joining the antibody and agent such that the agent can be 5 transferred across the blood brain barrier in a pharmaceutically active form. In preferred embodiments, the attached substance is a neuropharmaceutical agent and the antibody or antibody fragment forms a fusion protein with the agent. The antibody has replaced the nominal 10 ligand, such as transferrin or receptor-binding derivatives of transferrin, in these embodiments.

In other embodiments, an antibody or antibody fragment, which is immunoreactive with a brain capillary endothelial cell receptor, and a second ligand, which is 15 reactive with the same or a different receptor type on the brain capillary endothelial cells, are joined together to form a fusion protein. The second ligand can be a second antibody or, more preferably, a nominal ligand such as transferrin, IGF1, IGF2 or insulin. Conversely, the two 20 ligands of the fusion protein can be two nominal ligands. These fusion proteins have the advantage of possessing the capacity of interacting twice as readily with brain capillary endothelial cell receptors than the fusion proteins of the present invention which have only one 25 ligand. These fusion proteins can be linked by either genetic or chemical conjugation means to neuropharmaceutical agents for transferal of these agents across the blood brain barrier in a pharmaceutically active form.

30 When fusion proteins comprising an antibody and a second ligand are used, the range of neuropharmaceutical agents that can be transferred across the blood brain barrier is markedly increased. In addition to proteins and polypeptides, other substances that can be linked to the 35 fusion proteins include antibiotics, adrenergic agents,

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anticonvulsants, nucleotide analogs, chemotherapeutic agents, anti-inflammatory agents and anti-trauma agents used to treat or prevent a disease of the brain or central nervous system (CNS). Examples of antibiotics include  
5 amphotericin B, gentamicin sulfate and pyrimethamine. Examples of adrenergic agents (including blockers) include dopamine and atenolol. Examples of chemotherapeutic agents include adriamycin, methotrexate, cyclophosphamide, etoposide and carboplatin. An example of an anticonvulsant  
10 which can be used is valproate and an anti-trauma agent which can be used is superoxide dismutase. Nucleotide analogs which can be used include azidothymidine (hereinafter AZT), dideoxyinosine (ddI) and dideoxycytidine (ddC). Examples of anti-inflammatory agents include tumor  
15 necrosis factor (TNF) and transforming growth factor (TGF $\beta$ ).

The neuropharmaceutical agent can be linked to the antibody - second ligand fusion protein using chemical conjugation techniques. Generally, the link is made via an  
20 amine or a sulphydryl group. The link can be a cleavable link or non-cleavable link depending upon whether the neuropharmaceutical agent is more effective when released in its native form or whether the pharmaceutical activity of the agent can be maintained while linked to the fusion  
25 protein. The determination of whether to use a cleavable or non-cleavable linker can be made without undue experimentation by measuring the activity of the drug in both native and linked forms or for some drugs can be determined based on known activities of the drug in both  
30 the native and linked form.

For some cases involving the delivery of protein or peptide agents to the brain, release of the free protein or peptide may not be necessary if the biologically active portion of the protein or peptide agent is unaffected by  
35 its attachment to the fusion protein. As a result,

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antibody-protein or antibody-peptide conjugates can be constructed using noncleavable linkers.

Examples of non-cleavable linker systems which can be used in these embodiments include the carbodiimide (EDC), 5 the sulphydryl-maleimide, and the periodate systems. In the carbodiimide system, a water soluble carbodiimide reacts with carboxylic acid groups on proteins and activates the carboxyl group. The carboxyl group is coupled to an amino group of the second protein. The 10 result of this reaction is a noncleavable amide bond between two proteins.

In the sulphydryl-maleimide system, a sulphydryl group is introduced onto an amine group of one of the proteins using a compound such as Traut's reagent. The other protein 15 is reacted with an NHS ester (such as gamma-maleimidobutyric acid NHS ester (GMBS)) to form a maleimide derivative that is reactive with sulphydryl groups. The two modified proteins are then reacted to form a covalent linkage that is noncleavable.

20 Periodate coupling requires the presence of oligosaccharide groups on either the fusion protein carrier or the protein to be delivered. If these groups are available on the protein to be delivered (as in the case of horseradish peroxidase (HRP)), an active aldehyde is formed 25 on the protein to be delivered which can react with an amino group on the carrier. It is also possible to form active aldehyde groups from the carbohydrate groups present on antibody molecules. These groups can then be reacted with amino groups on the protein to be delivered generating 30 a stable conjugate. Alternatively, the periodate oxidized antibody can be reacted with a hydrazide derivative of a protein to be delivered which will also yield a stable conjugate.

35 Cleavable linkers can be used to link neuro-pharmaceutical agents which are to be deposited in the

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brain or when a non-cleavable linker alters the activity of a neuropharmaceutical agent. Examples of cleavable linkers include the acid labile linkers described in copending patent application Serial No. 07/308,960 filed February 6, 5 1989, the contents of which are hereby incorporated by reference. Acid labile linkers include disulfides such as N-succinimidyl-3-(2-pyridylthio) propionate (SPDP; Pharmacia), cis-aconitic acid, cis-carboxylic alkadienes, cis-carboxylic alkatrienes, and poly-maleic anhydrides. 10 Other cleavable linkers are linkers capable of attaching to primary alcohol groups. Examples of neuropharmaceutical agents which can be linked via a cleavable link include AZT, dDI, ddC, adriamycin, amphotericin B, pyrimethamine, valproate, methotrexate, cyclophosphamide, carboplatin and 15 superoxide dismutase. The noncleavable linkers used generally to link proteins to the antibody can also be used to link other neuropharmaceutical agents to the antibody.

SPDP is a heterobifunctional crosslinking reagent that introduces thiol-reactive groups into either the monoclonal 20 antibody or the neuropharmaceutical agent. The thiol-reactive group reacts with a free sulphhydryl group forming a disulfide bond.

In addition to covalent bonding, conjugates can be formed employing non-covalent bonds, such as those formed 25 with bifunctional antibodies, ionic bonds, hydrogen bonds, hydrophobic interactions, etc. The important consideration is that the conjugate bond be strong enough to result in passage of the conjugate through the blood-brain barrier.

Antibodies which can be used within this invention are 30 reactive with a receptor on a brain capillary endothelial cell. The term antibody is intended to encompass both polyclonal and monoclonal antibodies. The preferred antibody is a monoclonal antibody reactive with a brain capillary endothelial cell receptor such as a transferrin receptor. The term antibody is also intended to encompass

mixtures of more than one antibody reactive with a transferrin receptor (e.g., a cocktail of different types of monoclonal antibodies reactive with a transferrin receptor), each of which is joined to a neuropharmaceutical agent or another ligand to form a fusion protein. The term antibody is further intended to encompass whole antibodies, biologically functional fragments thereof, and chimeric antibodies comprising portions from more than one species, bifunctional antibodies, etc. Biologically functional antibody fragments which can be used are those fragments sufficient for binding of the antibody fragment to the brain capillary endothelial cell receptor to occur.

The chimeric antibodies can comprise portions derived from two different species (e.g.; human constant region and murine variable or binding region). The portions derived from two different species can be joined together chemically by conventional techniques or can be prepared as fusion proteins using genetic engineering techniques. In addition, DNA encoding the proteins of both the light chain and heavy chain portions of the chimeric antibody can be expressed together as fusion proteins.

Such chimeric antibodies can readily be adapted to being part of the fusion proteins of this invention. The DNA which contains the variable region coding sequence can be fused to DNA which contains the neuropharmaceutical agent coding sequence for subsequent expression as a fusion protein. Likewise, the DNA which contains the variable region coding sequence can be fused to DNA which contains the coding sequence of a second ligand, if such an expressed fusion protein is desired. The chimeric antibodies comprising constant and variable region portions from two different species can easily be converted to fusion proteins of this invention by inserting DNA encoding a neuropharmaceutical agent or DNA encoding another ligand after a specific portion of constant region encoding DNA.

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The subsequently expressed fusion protein will then contain the variable region from one species, a desired portion of the constant region from another species and a second ligand or the agent to be transferred across the blood

5 brain barrier.

Monoclonal antibodies reactive with at least a portion of the transferrin receptor can be obtained (e.g., OX-26, B3/25 (Omary *et al.* (1980) Nature **286**: 888-891), T56/14

(Gatter *et al.* (1983) J. Clin. Path. **36**: 539-545; Jefferies

10 *et al.* Immunology (1985) **54**: 333-341), OKT-9 (Sutherland *et*

al. (1981) Proc. Natl. Acad. Sci. USA **78**: 4515-4519), L5.1

(Rovera, C. (1982) Blood **59**: 671-678), 5E-9 (Haynes *et*

15 *al.* (1981) J. Immunol. **127**: 347-351), RI7 217 (Trowbridge *et*

*al.* Proc. Natl. Acad. Sci. USA **78**: 3039 (1981) and T58/30

15 (Omary *et al.* cited supra) or can be produced using somatic cell hybridization techniques (Kohler and Milstein

(1975) Nature **256**: 495-497) or by other techniques. In a

typical hybridization procedure, a crude or purified protein or peptide comprising at least a portion of the

20 transferrin receptor can be used as the immunogen. An animal is vaccinated with the immunogen to obtain

anti-transferrin receptor antibody-producing spleen cells.

The species of animal immunized will vary depending on the species of monoclonal antibody desired. An antibody-

25 producing cell is fused with an immortalizing cell (e.g. myeloma cell) to create a hybridoma capable of secreting anti-transferrin receptor antibodies. The unfused

residual antibody-producing cells and immortalizing cells

are eliminated. Hybridomas producing the anti-transferrin

30 receptor antibodies are selected using conventional techniques and the selected anti-transferrin receptor

antibody producing hybridomas are cloned and cultured.

Similar somatic cell hybridization techniques can be used

to produce hybridomas that secrete monoclonal antibodies

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immunoreactive with other brain capillary endothelial cell receptors.

Polyclonal antibodies can be prepared by immunizing an animal with a crude or purified protein or peptide

5 comprising at least a portion of a transferrin receptor or of another brain capillary endothelial cell receptor. The animal is maintained under conditions whereby antibodies reactive with a transferrin receptor are produced. Blood is collected from the animal upon reaching a desired titer  
10 of antibodies. The serum containing the polyclonal antibodies (antisera) is separated from the other blood components. The polyclonal antibody-containing serum can optionally be further separated into fractions of particular types of antibodies (e.g. IgG, IgM).

15 The ligand-neuropharmaceutical agent fusion proteins or conjugates can be administered orally, by subcutaneous or other injection, intravenously, intra-arterially, intramuscularly, parenterally, transdermally, nasally or rectally. The form and concentration in which the  
20 conjugate is administered (e.g., capsule, tablet, solution, emulsion) will depend at least in part on the route by which it is administered.

A therapeutically effective amount of a ligand-neuropharmaceutical agent fusion protein or  
25 conjugate is that amount necessary to significantly reduce or eliminate symptoms associated with a particular neurological disorder. The therapeutically effective amount will be determined on an individual basis and will be based, at least in part, on consideration of the  
30 individual's size, the severity of symptoms to be treated, the result sought, the specific ligand, etc. Thus, the therapeutically effective amount can be determined by one of ordinary skill in the art employing such factors and using no more than routine experimentation.

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The present invention will be illustrated by the following examples:

**EXAMPLE 1- In Vitro Binding of Murine Monoclonal Antibodies to Human Brain Endothelial Cells**

5        Two murine monoclonal antibodies, B3/25 and T58/30, described by Trowbridge (U.S. Patent 4,434,156 issued February 28, 1984, and Nature 294, pp. 171-173 (1981)), the contents of both are hereby incorporated by reference, which recognize the human transferrin receptor were tested

10      for their ability to bind to human brain capillary endothelial cells. Hybridoma cell lines which produce B3/25 and T58/30 antibodies were obtained from the American Type Culture Collection (ATCC) in Rockville, Maryland, and grown in DMEM medium supplemented with 2.0 mM glutamine,

15      10.0 mM HEPES (pH 7.2), 100 µM non-essential amino acids and 10% heat-inactivated fetal calf serum. The hybridoma cultures were scaled-up in 225 cm<sup>2</sup> T-flasks for the production of milligram quantities of IgG antibody. The hybridoma supernatants were concentrated 50x using vacuum

20      dialysis and applied to a protein-A sepharose column using the BioRad MAPS buffer system. Purified antibody was eluted from the column, dialyzed against 0.1 M sodium phosphate (pH 8.0), concentrated and stored in aliquots at -20°C.

25      Primary cultures of human brain endothelial cells were grown in flat-bottom 96-well plates until five days post-confluence. The cells were then fixed using 3.0% buffered formalin and the plate blocked with 1.0% bovine serum albumin (BSA) in Dulbecco's phosphate buffered saline

30      (DPBS). Aliquots (100 µl) of the B3/25 or T58/30 antibodies, either in the form of culture supernatants or purified protein, were then added to the wells (antibody concentrations were in the range of 1-50 µg/ml). Antibody

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which had specifically bound to the fixed cells was detected using a biotin-labeled polyclonal goat-anti-mouse IgG antisera followed by a biotinylated horseradish peroxidase (HRP)/avidin mixture (Avidin Biotin Complex technique). Positive wells were determined using a Titertek Multiscan Enzyme Linked Immunosorbent Assay (ELISA) plate reader. The results showed that both antibodies bind to human brain capillary endothelial cells with the T58/30 antibody exhibiting a higher level of binding.

These same antibodies were also tested for binding to human brain capillaries using sections of human brain tissue that were fresh frozen (without fixation), sectioned on a cryostat (section thickness was 5-20  $\mu$ m), placed on glass slides and fixed in acetone (10 minutes at room temperature). These sections were then stored at -20°C prior to use.

The slides containing the human brain sections were allowed to come to room temperature prior to use. The sections were then rehydrated in DPBS and incubated in methanol containing 0.3%  $H_2O_2$  to block endogenous peroxidate activity. The sections were blocked for fifteen minutes in a solution containing 0.2% non-fat dry milk and 0.2% methylmannopyranoside. B3/25 and T58/30 antibodies, purified as discussed above, were applied to the sections at a concentration of 5-50  $\mu$ g/ml and incubated at room temperature for one to two hours. Antibody that specifically bound to the tissue was detected using the Avidin-Biotin Complex (ABC) technique as described above for the ELISA assay. Staining of capillaries in the human brain sections was observed with both the B3/25 and T58/30 antibodies. The T58/30 antibody also displayed some binding to the white matter of the brain cortex.

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**EXAMPLE 2- In-Vitro Binding of Murine Monoclonal Antibody OX-26 to Rat Transferrin Receptor**

The OX-26 murine antibody, which recognizes the rat transferrin receptor, has been shown in vivo to bind to 5 brain capillary endothelial cells (Jeffries *et al.*, cited supra). The murine hybridoma line which produces the OX-26 murine antibody was obtained and the hybridoma cell line was grown in RPMI 1640 medium supplemented with 2.0 mM glutamine and 10% heat-inactivated fetal calf serum. The 10 OX-26 antibody was purified using the affinity chromatography technique described in Example 1.

The purified antibody was tested in vitro as described for the anti-human transferrin receptor antibodies in Example 1 to determine whether it would bind to brain 15 capillaries in fresh frozen, acetone-fixed rat brain sections. The results showed that the OX-26 anti-transferrin receptor antibody did bind to capillaries in rat brain sections in vitro.

**EXAMPLE 3- In-Vivo Binding of OX-26 Murine Monoclonal 20 Antibody to Rat Transferrin Receptor**

Dose Range

The anti-rat transferrin receptor antibody OX-26 was tested in vivo by injecting purified antibody (purification as described in Example 1) into female Sprague-Dawley rats 25 (100-150 gm) via the tail vein. Prior to injection, the rats were anesthetized with halothane. The samples, ranging from 2.0 mg to 0.05 mg of antibody/rat were injected into the tail vein in 400  $\mu$ l aliquots. All doses were tested in duplicate animals. One hour post-injection, 30 the animals were sacrificed and perfused through the heart with DPBS to clear the blood from the organs. Immediately after the perfusion was completed, the brain was removed and quick frozen in liquid nitrogen. The frozen brain was

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then sectioned (30-50  $\mu$ m) on a cryostat and the sections placed on glass microscope slides. The brain sections were air dried at room temperature one to two hours before fixation in acetone (10 minutes at room temperature).

5 After this treatment the sections could be stored at -20°C.

The OX-26 antibody was localized in the brain sections using immunohistochemistry as described above for the *in vitro* experiments in Example 1. The addition of the primary antibody was unnecessary in that it is present in 10 the brain sections. The results indicated that the OX-26 antibody binds to rat brain capillary endothelial cells and that doses of as little as 50  $\mu$ g result in detectable levels of antibody in the brain using the methods described herein. Doses above 0.5 mg did not appear to show 15 significantly more antibody binding to the endothelial cells, suggesting that the sites for antibody binding may be saturated. No specific binding to capillary endothelium was detected in the liver, kidney, heart, spleen or lung.

A non-specific antibody of the same subclass as OX-26 20 (IgG 2a) was also tested *in vivo* to show that the binding of OX-26 to rat brain endothelial cells that has been observed is specific to the OX-26 antibody. 0.5 mg of the control antibody was injected per rat as described above. The results indicate that the staining pattern observed 25 with the OX-26 antibody is specific to that antibody.

Time Course

After establishing that the OX-26 antibody is detectable in the rat brain capillaries after *in vivo* administration, the time frame in which this binding 30 occurred was determined. Using 0.5 mg of purified OX-26 antibody as the standard dose, brain sections taken from animals sacrificed 5 minutes, 15 minutes, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours post-injection were examined for the presence of OX-26 antibody. All doses were

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administered in 400  $\mu$ l aliquots and each time point was tested in duplicate animals. Samples were injected and the rats were processed at the various times postinjection as described above in the dose range section.

5 The results showed that the OX-26 antibody can be detected in or on the rat brain capillary endothelial cells as early as five minutes and as late as 24 hours post-injection. At 4 and 8 hours post-injection, the staining pattern of the antibody is very punctate  
10 suggesting that the antibody has accumulated in vesicular compartments either in endothelial or perivascular cells.

**EXAMPLE 4- The Use of a Conjugate of OX-26 Murine Monoclonal Antibody for Transferring Horseradish Peroxidase Across the Blood Brain Barrier**

15 Horseradish Peroxidase (HRP; 40 kD) was chosen as a compound to be delivered to the brain because it is similar in size to several therapeutic agents and it can be easily detected in the brain using an enzymatic assay. HRP was conjugated to the OX-26 antibody using a non-cleavable  
20 periodate linkage and the ability of the antibody to function as a carrier of compounds to the brain was examined. The antibody conjugate was tested in vivo to determine if the antibody could deliver HRP to the brain.

The antibody (10 mg) was first dialyzed overnight  
25 against 0.01 M sodium bicarbonate (pH 9.0). The HRP (10 mg) was dissolved in 2.5 ml deionized water, 0.1 M sodium periodate (160  $\mu$ l) was added and the mixture was incubated for five minutes at room temperature. Ethylene glycol (250  $\mu$ l) was added to the HRP solution followed by an additional  
30 five minute incubation. This solution was then dialyzed overnight against 1.0 mM sodium acetate buffer (pH 4.4). To the dialyzed OX-26 antibody (2.0 ml, 5.08 mg/ml) was added 200  $\mu$ l of 1.0 M sodium bicarbonate buffer, pH 9.5 and

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1.25 ml of the dialyzed HRP solution. This mixture was incubated in the dark for two hours followed by the addition of 100  $\mu$ l of 10 mg/ml sodium borohydride. The resulting mixture was incubated for two additional hours in

5 the dark at 4°C. The protein was precipitated from the solution by the addition of an equal volume of saturated ammonium sulfate and resuspended in a minimal volume of water. Free antibody was removed from the mixture by chromatography on a concanavalin A-sepharose column (a

10 column which binds HRP and the HRP-antibody conjugate and allows the free antibody to pass through). The free HRP was removed by chromatography on a protein A-sepharose column which retains the antibody-HRP conjugate. The final product had an HRP/antibody ratio of 4/1.

15 A time course experiment identical to that described in Example 3 was performed using the antibody-HRP conjugate. The antibody-HRP conjugate (0.5 mg) was injected in a 400  $\mu$ l aliquot/rat. The animals were sacrificed at the various times post-injection and the

20 brains processed as described above in Example 3. The antibody HRP conjugate was localized in the brain either by staining for antibody immunohistochemically as described in Example 1 or by directly staining the brain sections for the presence of HRP. To detect HRP, the slides were first

25 allowed to come to room temperature before incubating in methanol for thirty minutes. The brain sections were then washed in DPBS and reacted with 3,3'-diamino benzidine (DAB), the substrate for HRP. The results showed that the OX-26 antibody HRP conjugate binds to rat brain capillary

30 endothelial cells in a manner identical to that of the unconjugated antibody. The punctate staining 4-8 hours after injection which was seen with the antibody alone is also seen with the antibody conjugate, suggesting that the conjugate can also be going into the pericytes on the

35 abluminal side of the blood brain barrier. Taken together,

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these results indicate that the OX-26 antibody can deliver a protein molecule of at least 40 KD to the brain.

**EXAMPLE 5- The In-Vivo Delivery of Adriamycin to the Brain by Murine Monoclonal Antibody OX-26**

5 A non-cleavable linker system similar to that used in Example 4, was used to couple the chemotherapeutic drug adriamycin to the OX-26 antibody. The availability of antibodies that can detect adriamycin as well as the system previously described in Example 1 for detecting the  
10 antibody carrier allowed the use of immunohistochemical techniques for monitoring the localization of the antibody carrier as well as the delivery of adriamycin to the brain.

To conjugate adriamycin to the antibody, the drug (10 mg in 0.5 ml DPBS) was oxidized by the addition of 200  $\mu$ l  
15 of 0.1 M sodium periodate. This mixture was incubated for one hour at room temperature in the dark. The reaction was quenched by the addition of 200  $\mu$ l of ethylene glycol followed by a five minute incubation. The OX-26 antibody (5.0 mg in 0.5 ml of carbonate buffer (pH 9.5)) was added  
20 to the oxidized adriamycin and incubated at room temperature for one hour. Sodium borohydride (100  $\mu$ l of 10 mg/ml) was added and the mixture was incubated for an additional two hours at room temperature. The free adriamycin was separated from the OX-26 antibody-  
25 adriamycin conjugate by chromatography on a PD-10 column. The adriamycin/OX-26 antibody ratio within the conjugate was 2/1 for this particular batch of conjugate.

The effectiveness of the OX-26 antibody as a carrier for delivering adriamycin to the brain was determined by  
30 administering 0.5 mg of the antibody-adriamycin conjugate in a 400  $\mu$ l aliquot per rat by injection via the tail vein. One hour post-injection, the rat was sacrificed and the

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brain processed as described in Example 1. All injections were performed in duplicate. As a control, 400  $\mu$ g of free adriamycin in a 400  $\mu$ l aliquot was also injected into a rat. Immunohistochemistry was used to 5 detect both the carrier OX-26 antibody and the adriamycin in the rat brain sections. In the case of adriamycin, polyclonal rabbit anti-adriamycin antisera was applied to the sections followed by a biotinylated goat anti-rabbit IgG antisera. This was then followed by the addition of a 10 biotinylated HRP/avidin mixture and enzymatic detection of HRP.

The results indicate that both the OX-26 antibody and the conjugated adriamycin localized to the rat brain capillary endothelial cells after in vivo administration. 15 There is no evidence that free adriamycin binds to brain capillary endothelial cells or enters the brain.

An adriamycin-OX-26 conjugate coupled via a carbodiimide linkage was also synthesized (drug/ antibody ratio of 10/1) and tested in vivo. The results of this 20 experiment were essentially identical to that obtained with the periodate-linked antibody-drug conjugate. In both cases, staining for the antibody carrier was quite strong and was visualized in the capillaries in all areas of the brain. This staining was evenly distributed along the 25 capillaries. Staining for adriamycin was less intense but again was seen in capillaries throughout the brain. Some punctate staining was observed which suggests accumulation in pericytes which lie on the brain side of the blood-brain barrier.

30 EXAMPLE 6- In Vivo Delivery of Methotrexate to the Brain by Murine Monoclonal Antibody OX-26.

A noncleavable carbodiimide linkage was used to couple methotrexate to the OX-26 murine monoclonal antibody. A

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system analogous to that described in Example 5 was used to monitor the delivery of both the methotrexate and the carrier antibody to the brain capillary endothelial cells.

Methotrexate was coupled to murine monoclonal antibody 5 OX-26 via its active ester. Briefly, 81 mg (0.178 mM) of methotrexate (Aldrich) was stirred with 21 mg (0.182 mM) of N-hydroxysuccinimide (Aldrich) in 3 ml of dimethylformamide (DMF) at 4°C. Ethyl-3-dimethylaminopropyl-carbodiimide (180 mg, EDC; 0.52 mM) was added to this solution and the reaction 10 mixture was stirred overnight. The crude ester was purified from the reaction by-products by flash chromatography over silica gel 60 (Merck) using a solution of 10% methanol in chloroform as an eluant. The purified active ester fractions were pooled and concentrated to 15 dryness. The ester was dissolved in 1 ml of DMF and stored at -20°C until use. 50 mg (50%) of active ester was recovered as determined by  $A_{372}$  ( $\epsilon_{372}=7200$ ).

A solution of OX-26 containing 2.1 mg (14 nmoles) of antibody in 0.9 ml of 0.1 M phosphate (pH 8.0) was thawed 20 to 4°C. To this stirred antibody solution was added 1.4  $\mu$ l (140 nmoles) of the active ester prepared as described above. After 16 hours at 4°C, the mixture was chromatographed over Sephadex PD-10 column (Pharmacia) using phosphate buffered saline (PBS) to separate conjugate 25 from free drug. The fractions containing the antibody-methotrexate conjugate were pooled. Antibody and drug concentration were determined spectrophotometrically as described by Endo *et al.* (*Cancer Research* (1988) 48: 3330-3335). The final conjugate contained 7 30 methotrexates/antibody.

The ability of the OX-26 monoclonal antibody to deliver methotrexate to the rat brain capillary endothelial cells was tested *in vivo* by injecting 0.2 mg of conjugate (in 400  $\mu$ l) into each of two rats via the tail vein. The 35 animals were sacrificed one hour post-injection and the

-26-

brains processed for immunohistochemistry as described in Example 1. To detect methotrexate in the brain, a rabbit antisera raised against methotrexate was used as the primary antibody. A biotinylated goat-anti-rabbit antisera 5 in conjunction with a mixture of biotinylated HRP and avidin was then used to visualize methotrexate in the rat brain. The carrier antibody was detected as described previously.

The results of these experiments indicate that 10 methotrexate in the form of a conjugate with OX-26 does accumulate along or in the capillary endothelial cells of the brain. The staining observed for methotrexate is comparable in intensity to that seen for the carrier. The staining appears to be in all areas of the brain and is 15 evenly distributed along the capillaries.

#### EXAMPLE 7- Antibody Derivatives

The Fc portion of the OX-26 murine monoclonal antibody was removed to determine whether this would alter its localization to or uptake by the rat brain capillary 20 endothelial cells.  $F(ab)_2$  fragments of OX-26 were produced from intact IgG's via digestion with pepsin. A kit available from Pierce Chemical Co. contains the reagents and protocols for cleaving the antibody to obtain the fragments. The  $F(ab')_2$  fragment (0.2 mg doses) in 25 400  $\mu$ l aliquots were injected into rats via the tail vein. A time course experiment identical to that done with the intact antibody (Example 2) was then performed.  $F(ab')_2$  fragment was detected immunohistochemically using a goat anti-mouse  $F(ab')_2$  antisera followed by a biotinylated 30 rabbit anti-goat IgG antisera. A biotinylated HRP/avidin mixture was added and the antibody complex was visualized using an HRP enzymatic assay. The results indicate that

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the F(ab)<sub>2</sub> fragment of the OX-26 antibody binds to the capillary endothelial cells of the rat brain.

**EXAMPLE 8 - Measurement of OX-26 in Brain Tissue**

To quantitate the amount of OX-26 which accumulates in the brain, radioactively-labelled antibody was injected into rats via the tail vein. Antibodies were labelled with either <sup>14</sup>C-acetic anhydride or <sup>3</sup>H-succinimidyl propionate essentially as described in Kummer, U., Methods in Enzymology, 121: 670-678 (1986), Mondelaro, R.C., and Rueckert, R.R., J. of Biological Chemistry, 250: 1413-1421 (1975), hereby incorporated by reference. For all experiments, the radiolabelled compounds were injected as a 400  $\mu$ l bolus into the tail vein of female Sprague-Dawley rats (100-125 gms) under Halothane anesthesia and the animals were sacrificed at the appropriate time post-injection using a lethal dose of anesthetic. A <sup>3</sup>H-labelled IgG2a control antibody was co-injected with the <sup>14</sup>C-labelled OX-26 to serve as a control for non-specific radioactivity in the brain due to residual blood. At the appropriate time post-injection, animals were sacrificed and the brains were removed immediately and homogenized in 5 ml of 0.5% sodium dodecylsulfate using an Omni-mixer. An aliquot of the homogenate was incubated overnight with 2 ml of Soluene 350 tissue solubilizer prior to liquid scintillation counting. All data were collected as disintegrations per minute (dpm). Blood samples were centrifuged to pellet red blood cells (which do not display significant binding of radiolabelled materials) and the radioactivity in an aliquot of serum determined using liquid scintillation counting.

The amount of antibody associated with the brain was determined at various times post-injection to examine the pharmacokinetics of brain uptake. In addition, the amount of labelled antibody in the blood was measured so that the

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rate of clearance from the bloodstream could be determined. This information was also used to calculate the amount of radioactivity in the brain due to blood contamination, which was then subtracted from the total to give the amount 5 of antibody that is specifically associated with the brain.

A peak level of  $^{14}\text{C}$ -labelled OX-26 corresponding to approximately 0.9% of the injected dose was reached in the brain between 1 and 4 hours post-injection as illustrated in Figure 1 (with the values shown as means plus or minus 10 standard error of the mean (SEM) and N=3 rats per time point). The amount of radioactivity associated with the brain decreased steadily from 4 to 48 hours post-injection, at which point it leveled off at approximately 0.3% of the injected dose. The accumulation of OX-26 in the brain was 15 significantly reduced by the addition of unlabelled monoclonal antibody (0.5 or 2.0 mg in the bolus injection). As an additional control, a  $^3\text{H}$ -IgG2a control antibody was co-injected with the 20  $^{14}\text{C}$ -OX-26. The control antibody did not accumulate in the brain and represented the blood contamination of the brain.

In contrast to the levels in the brain, the blood level of OX-26 dropped quite dramatically immediately after injection such that by 1 hour post-injection, the percent 25 of injected dose in 55  $\mu\text{l}$  of blood (the volume of blood associated with the brain) was approximately 0.16% as illustrated in Figure 1. This corresponds to a value of approximately 20% of the injected dose in the total blood volume of the rat. Extraction of total IgG from serum 30 followed by polyacrylamide gel electrophoresis (PAGE) and autoradiography did not reveal detectable levels of OX-26 degradation indicating that the antibody remains intact in the blood as long as 48 hours after injection.

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EXAMPLE 9 - Distribution of OX-26 in Brain Parenchyma and Capillaries

To demonstrate that anti-transferrin receptor antibody accumulates in the brain parenchyma, homogenates of brains taken from animals injected with labelled OX-26 were depleted of capillaries by centrifugation through dextran to yield a brain tissue supernatant and a capillary pellet. Capillary depletion experiments followed the procedure of Triguero, *et al.*, *J. of Neurochemistry*, 54: 1882-1888 (1990), hereby incorporated by reference. As for the brain uptake experiments of Example 8, the radiolabelled compounds were injected as a 400  $\mu$ l bolus into the tail vein of female Sprague-Dawley rats (100-125 gm) under Halothane anesthesia and the animals were sacrificed at the appropriate time post-injection using a lethal dose of anesthetic. A  $^3$ H-labelled IgG 2a control antibody was co-injected with the  $^{14}$ C-labelled OX-26 to serve as a control for non-specific radioactivity in the brain due to residual blood. After sacrifice, the brains were removed and kept on ice. After an initial mincing, the brains were homogenized by hand (8-10 strokes) in 3.5 ml of ice cold physiologic buffer (100 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 14.5 mM HEPES, 10 mM D-glucose, pH 7.4). Four ml of 26% dextran solution in buffer was added and homogenization was continued (3 strokes). After removing an aliquot of the homogenate, the remainder was spun at 7200 rpm in a swinging bucket rotor. The resulting supernatant was carefully removed from the capillary pellet. The entire capillary pellet and aliquots of the homogenate and supernatant were incubated overnight with 2 ml of Soluene 350 prior to liquid scintillation counting. This method removes greater than 90% of the vasculature from the brain homogenate (Triguero *et al.*, cited supra).

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A comparison of the relative amounts of radioactivity in the different brain fractions as a function of time indicates whether transcytosis of the labelled antibody has occurred. The amount of OX-26 in total brain homogenate, 5 the brain parenchyma fraction and the brain capillary fraction at an early time (30 minutes) and a later time (24 hours) post-injection is illustrated in Figure 2. The values in Figure 2 are shown as means $\pm$ SEM with N=3 rats per time point. At the 30 minute time point, more of the 10 radiolabelled antibody is associated with the capillary fraction than with the brain parenchyma fraction (0.36% of the injected dose (%ID) and 0.23% ID, respectively). By 24 hours post-injection, the distribution is reversed and the majority of the radioactivity (0.36% ID) is in the 15 parenchymal fraction as compared to the capillary fraction (0.12% ID). The redistribution of the radiolabelled OX-26 from the capillary fraction to the parenchyma fraction is consistent with the time dependent migration of the anti-transferrin receptor antibody across the blood-brain 20 barrier.

**EXAMPLE 10 - Biodistribution and Brain Uptake of Anti-Human Transferrin Receptor Antibodies in Cynomolgous Monkeys**

A collection of 32 murine monoclonal antibodies which recognize various epitopes on the human transferrin 25 receptor were examined for reactivity with brain capillary endothelial cells in sections from human, monkey (cynomolgous), rat and rabbit brain samples by the immunohistochemical methods described in Example 1. These antibodies were obtained from Dr. Ian Trowbridge of the 30 Salk Institute, LaJolla, CA. All 32 antibodies displayed some reactivity with human brain endothelial cells. Two antibodies reacted very weakly with rabbit brain capillaries and none reacted with rat. While 21 of the

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antibodies reacted with monkey brain capillaries, only 2 displayed strong reactivity comparable to that seen with human brain capillaries. These 2 antibodies are herewithin referred to as 128.1 and Z35.2.

5 These antibodies were used to determine the tissue distribution and blood clearance of the  $^{14}\text{C}$ -labelled anti-human transferrin receptor antibodies 128.1 and Z35.2 in 2 male cynomolgous monkeys. 128.1 or Z35.2 was administered concurrently with a  $^3\text{H}$ -labelled control IgG to 10 one of the monkeys with an intravenous catheter. During the course of the study, blood samples were collected to determine the clearance of the antibodies from the circulation. At 24 hours post-injection, the animals were euthanized and selected organs and representative tissues 15 were collected for the determination of isotope distribution and clearance by combustion. In addition, samples from different regions of the brain were processed as described for the capillary depletion experiments in Example 9 to determine whether the antibodies had crossed 20 the blood-brain barrier. The results of the capillary depletion experiments were performed on samples from the cortex, frontal cortex, cerebellum and striatum. All samples had greater than 90% of the 128.1 or Z35.2 in the brain parenchyma, suggesting that the antibodies crossed 25 the blood-brain barrier. The levels of the control antibody in the same samples were from 5 to 10-fold lower. Using the average brain homogenate value for dpm/G tissue, the percent injected dose of 128.1 in the whole brain is approximately 0.2-0.3%. This compares to a value of 30 0.3-0.5% for OX-26 in the rat at 24 hours post-injection. A comparison of the ratios of 128.1 to the control antibody for various organs is illustrated in Figure 3. Similar results were obtained for Z35.2. These results suggest that 128.1 is preferentially taken up by the brain as 35 compared to control antibody. For the majority of organs

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and tissues tested, the ratio of 128.1 to control is less than 2.

**EXAMPLE 11 - Construction of a NGF-IgG3 Hinge-Transferrin Fusion Gene, Expression of the NGF-IgG3 Hinge-Transferrin 5 Fusion Gene as a Fusion Protein and Assay of the Fusion Protein Constituents**

A fusion protein comprised of human NGF and human transferrin was constructed using the human IgG3 hinge region as a linker between the NGF and transferrin 10 polypeptides. The IgG3 hinge region, which is about 60 amino acids long and includes a number of cysteine residues, was chosen as a connector between the NGF and transferrin polypeptides. The potential for disulfide formation within the immunoglobulin hinge region was 15 envisioned as increasing the probability of allowing the dimerization of the NGF polypeptides into a more native NGF configuration.

Preparation of a Bacterial Vector for Insertion of the NGF Gene.

20 Bacterial plasmid pAT3442 (Fig. 4) was used as the starting plasmid for construction of the NGF-hinge-transferrin gene fusion. It contained human genomic DNA encoding the IgG3 CH1 (constant region 1 of the heavy chain) and hinge region and the human transferrin cDNA. 25 This plasmid was constructed by the following procedure.

Bacterial plasmid pAT3442 was derived from vector pAT153 ("Practical Guide to Molecular Cloning, 1984, Bernard Perbal, John Wiley Publisher). pAT153 was first modified to remove the EcoRI site by cleavage of this 30 vector with EcoRI, filling in of the 5' overhang regions with the use of DNA polymerase, and religation. This derivative was designated pAT153.7.

A SalI-BamHI fragment containing the sequence coding for human IgG3 constant region and the hinge region with its associated introns was isolated from phage lambda libraries as described in Dangl, J.L., (1986 Dissertation, 5 Stanford University, Stanford, CA). A large portion of the untranslated region was eliminated from the 3' end of the IgG3 gene by cleavage with PvuII (which cleaves multiple times within this region) and religation. This fragment was further modified by site directed mutagenesis 10 to contain a PvuII site at the 5' end of the CH2 region; cleavage with SmaI and introduction of an EcoRI linker resulted in the SmaI site in the CH3 region being joined to the SmaI site 0.6kb upstream of the BamHI site with an EcoRI site separating them. This SalI - BamHI fragment 15 with the PvuII and EcoRI sites was cloned into SalI and BamHI cleaved pAT153.7 (pAT153 with its EcoRI site deleted by filling in; also called pAT3404) yielding pAT3408.

A 2.4 kb Pst I fragment containing the human transferrin cDNA sequence was isolated from clone Tf 20 (U.S. Patent No. 5,026,651) and cloned into the PstI site of pBluescript II KS [Stratagene] creating pKS3436. A PvuII site was introduced at the 3' end of the leader sequence of the transferrin by standard site-directed mutagenesis procedures, thereby creating pKS3438. An 25 EcoRI site beyond the 3' end of the transferrin gene and the newly introduced PvuII site were used to clone the 2.4kb fragment containing the transferrin coding sequence with its associated polyA site into pAT3408; transferrin thereby replaced the CH2 and CH3 domains of IgG3. As a 30 consequence of this manipulation, a nucleotide sequence encoding the amino acid sequence ala-ala precedes the mature transferrin coding sequence. Approximately 600 bp of the region 3' of IgG3 adjacent to the BamHI site were adjacent to the 3' end of the transferrin gene. The 35 resulting plasmid was designated pAT3442 (Figure 4).

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The unique Eco RV site downstream of the 3' untranslated IgG3 sequences in plasmid pAT3442 was converted to an XbaI site by digestion with EcoRV and ligation of a synthetic linker containing an XbaI 5 restriction site in order to facilitate future cloning into appropriate mammalian vectors. A clone containing the new XbaI site was designated pATX. A map of the CH1-hinge-transferrin (CH1-hinge-Tf) region of pATX is shown in Figure 5A.

10 The CH1 coding sequence in pATX was then replaced with the NGF gene. pATX was modified using polymerase chain reaction (PCR) techniques in two steps. The 5' PCR primer, HTF-1 (shown below), contained SalI and XbaI cloning sites near its 5' end, and 14 bases complementary 15 to the first intron of the hinge region at its 3' end.

SalI      XbaI      first hinge intron  
HTF-1      5'-GG GTCGAC CTCCGAG GGT GAG AGG CCA GC-3'  
(SEQ.ID.NO.1)

The 3' primer (HTF-2) was complementary to a sequence 20 within the first intron of the hinge region, approximately 400 nucleotides downstream of the 5' primer, and included a BglII cloning site.

first hinge intron BglII  
HTF-2      5'-GGAGTTACTC AGATCT GGGAAAG-3'  
(SEQ.ID.NO.2)

25 The primers were combined with pATX template DNA and, following the PCR procedures, the 400 bp amplified fragment was gel purified, digested with SalI and BglII, and cloned into pATX which had been digested to completion 30 with SalI and partially with BglII (to cleave at one of two BglII sites of this plasmid). The ligated sample was transformed into *E.coli* DH1 cells and a clone having the 400 bp ligated fragment in place of the CH1 region was

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identified by restriction digestion analysis. This clone was designated pATXX; a map of the hinge-transferrin region of pATXX is shown in Figure 5B.

Isolation and Cloning of the NGF gene.

5 The pre-pro form of the NGF gene, which is about twice the size of the mature  $\beta$ -NGF coding sequence and contains the signals for protein secretion and protein folding, was amplified by PCR techniques from human erythrocyte genomic DNA (purchased from Clontech, Palo 10 Alto, CA) using the following primers.

PNGF 1 (5' Primer)  
HindIII Xhol XbaI Start  
5'-G AAGCTT CTCGAG TCTAGA CCAGGTGCATAGCGTA ATG TCC-3'  
(SEQ.ID.NO.3)

15 PNGF 2 (3' Primer)  
SalI XbaI Start  
5'-C GTCGAC CTCGAG TCTCACAGCCTTCCTGCTGAGC-3'  
(SEQ.ID.NO.4)

PNGF1 and PNGF2 were partially homologous to sequences at 20 the 5' and 3' ends of the pre-pro NGF gene, respectively, and were devised to create XbaI cloning sites at either end of the NGF gene. The 5' primer additionally contained an XbaI cloning site.

Using the PNGF1 and PNGF2 primers, a fragment of 25 approximately 800 bp containing the pre-pro NGF gene was amplified. The resulting DNA was digested with XbaI, and the (approximately) 800 bp fragment was gel purified and ligated into XbaI digested pATXX. A clone designated pATXXNGF was identified which had the pre-pro NGF gene 30 inserted upstream and adjacent to the IgG3 hinge region in the same orientation as the transferrin gene thereby creating an NGF-IgG3 hinge-transferrin (NHT) gene fusion.

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A map of this region of pATXXNGF is shown in Figure 5C. (The XbaI site 3' to the pre-pro NGF coding region of pATXXNGF encodes a leu-glu which precedes the IgG3 hinge region).

5 A partial DNA sequence of the junctions between the  
NGF-hinge and hinge-transferrin sequences were determined  
in order to verify the sequence of the primers and to  
confirm the correct reading frame within the newly formed  
gene fusion. The determined sequences revealed one  
10 nucleotide change, a G to A transition, which resulted in  
an arginine to glutamine change at amino acid position 80  
in the pre-pro portion of NGF.

## Reconstruction of the NGF-Hinge-Transferrin Genetic Fusion in Mammalian Expression Vectors

15 The NGF-IgG3 hinge-transferrin gene fusion was cloned  
stepwise into mammalian expression vector pcDNA1/AMP  
(Invitrogen) for transfection into COS cells (ATCC  
Accession Number CRL 1651) using the following procedure.

The pre-pro NGF portion of the gene fusion was first amplified from pATXXNGF using PCR techniques. The 5' PCR primer, PNGF1, was described above and the 3' PCR primer, PNGF3, is shown below.

### PNGF3 (3' Primer)

25 SalI XbaI .  
 5'-C GTCGAC TCTAGA TTA TCTCACAGCCTTC-3'  
 Stop  
 (SEQ. ID. NO. 5)

Using the PNGF1 and PNGF3 primers, an approximately 800 bp fragment containing the pre-pro NGF gene was amplified 30 from pATXXNGF. The resulting DNA was digested with XbaI, ligated into XbaI digested bacterial vector pUC18 (Boehringer-Mannheim) and transformed into competent *E.coli* TOP10F cells (Invitrogen). Clones containing the NGF gene in either orientation were identified by

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restriction analysis and designated pUCNGF1 (clockwise orientation) and pUCNGF2 (counterclockwise orientation) (Figure 6).

The entire NHT gene fusion was then recreated in 5 pcDNAI/Amp using a three-part ligation. The first fragment, an approximately 600 bp fragment containing most of the NGF gene, was removed from pUCNGF2 by digestion with BamHI, which cleaves within the polylinker sequence upstream of the pre-pro NGF gene sequence, and Scal, which 10 cleaves near the 3' end of the NGF gene. The second fragment was a 4.7 kb Scal to XbaI fragment isolated from pATXXNGF which contained the remainder of the 3' end of the NGF gene, the hinge region and the entire transferrin gene. The third fragment was the pcDNAI/Amp vector which 15 had been digested within its polylinker sequence with BamHI and XbaI and gel purified. The three fragments were ligated together (Fig. 7) and transformed into *E.coli* cells. A plasmid containing all three fragments was identified and designated pcDNAI/AmpNHT. The DNA sequence 20 was again determined to verify the NGF and transferrin coding sequences. The determined sequence revealed a T to C transition in NGF which resulted in a valine to alanine change at amino acid 35, and three changes from the published transferrin nucleotide sequence that did not 25 result in amino acid changes.

Assay for NGF and Transferrin in the Fusion Protein Expressed in Mammalian Cells.

Expression plasmid pcDNAI/AmpNHT was transfected and transiently expressed in COS cells. The fusion protein in 30 culture supernatants was detected using anti-NGF antibodies, anti-transferrin antibodies, or purified transferrin receptor by standard ELISA procedures.

Briefly, capture antibody (anti-NGF or anti-transferrin), which was specific for either the NGF or

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transferrin portions of the fusion protein, was coated in the wells of a 96-well plate. The wells were washed (PBS-0.05% Tween), blocked with 1% bovine serum albumin (BSA), and supernatants from transfected COS cells were added to 5 the wells (typically in serial four-fold dilutions) and incubated for one hour at room temperature.

A detection antibody was chosen which would recognize the other portion of the fusion protein (either anti-Tf or anti-NGF) and was added to the wells on top of the fusion 10 protein. Bound antibody was detected after amplification of the signals by an avidin-biotin reaction using the Vectastain ABC kit (VectorLabs). Protein was quantitated by extrapolation from standard curves generated for known concentrations of NGF or transferrin.

15 Alternative ELISA procedures may be used to detect and quantitate the fusion proteins. For example, the capture antibody and detection antibody may recognize the same portion of the fusion protein.

Optimization of Expression of the NHT Fusion Proteins

20 In order to increase the level of expression of the NHT fusion protein, the translation initiation sequence immediately preceding the AUG start codon of the pre-pro NGF gene was modified using PCR techniques to incorporate a Kozak consensus sequence (Kozak, 1987, Nucl. Acid. Res. 25 15, 8125).

Two PCR primers were designed which were complementary to regions flanking the pre-pro NGF gene sequence. The 5' primer, p45.1, contained an SmaI restriction site as well as the sequence CCACC, the Kozak 30 sequence, which has been shown to be important for efficient translation in mammalian cells, immediately preceding the ATG initiation codon of the pre-pro NGF gene.

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p45.1

Smal      XbaI      Kozak  
5'-TCC CCCGGG TCTAGA CCAGGGTGCAT CCACCC ATGTCATGTTGTT-3'  
(SEQ.ID.NO.6)

5 The 3' primer, p16.1, was complementary to a sequence 3'  
to both the NGF coding sequence and the BamHI site in  
pUCNGF1.

p16.1

10      5'-AACAGCTATGACCATG-3'  
(SEQ.ID.NO.7)

By PCR techniques, the pre-pro NGF coding sequence,  
preceded by the Kozak translation initiation consensus  
sequence in p45.1, was amplified from pUCNGF1 using the  
15 p45.1 and p16.1 primers. The resulting DNA can be cloned  
into the mammalian expression vector, CD5lnegl.

One way to accomplish this cloning is to first insert  
the pre-pro NGF gene sequence into a bacterial vector, for  
example pGEM-2 (Promega), having compatible restriction  
20 sites for subsequent cloning into the mammalian expression  
vector. The amplified pre-pro NGF gene can be digested  
with Smal and BamHI and ligated into the polylinker of  
pGEM-2 between HincII and BamHI sites (since Smal and  
HincII produce blunt-ended fragments) thereby producing a  
25 clone designated pGEM-2/KNGF.

The NHT fusion is then recreated by a three-part  
ligation as follows. A HindIII-ScaI fragment from the  
pGEM-2/KNGF clone (containing most of the NGF coding  
region) is mixed with the ScaI-XbaI fragment from  
30 pcdNA1/AmpNHT (containing the C-terminal-most portion of  
the NGF gene, the IgG3 hinge and the transferrin coding  
sequence) and HindIII + XbaI digested pGEM-2 vector. The  
resulting vector has the entire NHT fusion between  
flanking XbaI sites (the 5' XbaI site from linker p45.1).

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This *Xba*I fragment is then cloned into the *Xba*I digested CD51neg1.

CD51neg1 contains a CMV promoter sequence, a CD5 leader sequence for secretion of a cloned protein, a 5 sequence encoding the hinge region and Fc portion of IgG1, and a polyadenylation signal sequence (Figure 8).

CD51neg1 (gift of Brian Seed, Massachusetts General Hospital) can be derived from plasmid pCDM8 (Invitrogen) by deletion of the 590 bp *Dra*I fragment within the SV40 10 intron sequence, the *Bam*HI-*Sfi*I fragment containing the polyoma origin of replication, and a 20 bp *Nhe*I-*Ssp*I fragment at the 3' end of M13 ori which was removed in order to eliminate the *Nhe*I site. In addition, CD51neg1 15 also contains the CD5 leader sequence (Genbank reference number X04391) adjacent to the IgG1 hinge-Fc region (Genbank reference number J00228) inserted between the *Xho*I (within polylinker) and *Eag*I (approximately 43 bp beyond polylinker) sites of pCDM8. The DNA sequence of the IgG1 hinge region between the *Xho*I and *Eag*I sites is 20 shown in Figure 9A-9B (Seq.I.D.NO.8). The CD5 leader sequence and IgG1 exons are indicated on this figure. The clone resulting from the insertion of the entire NHT 25 fusion into CD51neg1 is designated CD5KNHT.

Vector CD5KNHT was transfected into CHO cells and 25 expression of the fusion protein was assayed by ELISA as described above. Proteins containing both the transferrin and NGF sequences were expressed and detected in the culture supernatant.

Purification of NGF-Hinge-Transferrin Fusion Protein from 30 COS Cell Supernatants.

NGF-hinge-transferrin fusion protein present in the supernatant of transfected COS cells was purified using an anti-NGF affinity column. Medium from cells transfected with CD5KNHT was loaded overnight by gravity onto a

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Sepharose column to which rat anti-mouse NGF monoclonal antibody had been bound (e.g., antibody 1G3, Saffron *et al.*, *Brain Res.* 1989, **492**:245-254). This column had been pre-equilibrated with PBS. The columns were washed five times with 2 ml PBS, and NGF containing proteins were eluted with 10 ml of a 0.1 M glycine, 0.15M NaCl solution, pH 3.0. The elution was accomplished as five 2 ml fractions which were placed directly into tubes containing 50  $\mu$ l 1M  $\text{NH}_4\text{HCO}_3$  and 8  $\mu\text{g}/\text{ml}$   $\text{FeNH}_4$  citrate. The  $\text{OD}_{280}$  of 10 each of the fractions was then determined.

The affinity purified fractions contained a major band at MW 100kD on a reducing SDS-polyacrylamide gel. The band was recognized by anti-transferrin antibody and anti-NGF antibody.

**15 In vitro Competition Assay for Transferrin Receptor Binding Activity**

A critical attribute of the NHT fusion protein is its ability to bind to the transferrin receptor. Assays were performed to measure the affinity of the NHT fusion 20 protein for the human transferrin receptor by the ability of the fusion protein to compete with native transferrin for binding to the transferrin receptor.

Transferrin receptor was purified from freshly obtained full term human placentas using the procedure of 25 Turkewitz *et al.* (1988, *J. Biol. Chem.*, **263**: 8318-8325). Briefly, placental membranes were isolated and stored frozen at -80°C. Frozen membranes were thawed and extracted with detergent. Endogenous iron was chelated, greatly lowering the affinity of endogenous transferrin 30 for the receptor. The transferrin receptor was then purified on a human transferrin-Sepharose column. By gel analysis, the resulting receptor had the expected molecular weight, and the purity, as determined by scanning densitometry of the gel, was 98%. The purified

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transferrin receptor was immunoblotted using antibody 128.1 and another commercially obtained anti-human transferrin receptor antibody (Amersham RPN.511).

For the competition assay, microtiter wells were 5 coated with 100  $\mu$ l/well of 0.7  $\mu$ g/ml purified human placental transferrin receptor in coating buffer (10 mM sodium carbonate, pH 9.5) overnight at 4°C. Nonspecific binding was blocked by incubation with 200  $\mu$ l/well of 1% w/v bovine serum albumin (BSA) for 1 hour at 37°C. The 10 wells were then washed, and 100  $\mu$ l of a mixture of 4 nM  $^{125}$ I-transferrin (New England Nuclear) and varying 15 concentrations of competitor, either purified recombinant human transferrin or NHT fusion protein, were applied to each well and incubated for 1 hour at room temperature. After washing, the wells were individually counted using a gamma counter. The percent signal remaining versus competitor concentration was plotted and the point of 50% remaining signal was determined (Figure 10). The results demonstrated that the NGF-hinge-transferrin fusion protein 20 binds to the human transferrin receptor but has reduced affinity for this receptor compared to native human transferrin.

In vitro Assay for NGF Activity.

A clonal line of rat pheochromocytoma cells 25 (designated PC12) undergoes cessation of cell division and extensive outgrowth of neurite-like processes in the presence of NGF *in vitro*. This cell-based bioassay was used to assess whether the expressed NHT fusion protein has NGF activity by measuring its ability to stimulate 30 neurite outgrowth.

PC12 cells were grown in RPMI 1640 medium (Bio Whittaker) containing 5% fetal calf serum, 10% horse serum and 2 mM L-glutamine, in T75 flasks under 5% CO<sub>2</sub>. Ninety-six well plates were coated with 0.5  $\mu$ g/cm<sup>2</sup> bovine

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collagen IV at 50  $\mu$ l/well, air dried overnight and exposed to UV light for 20 minutes prior to use. Five ml of PC12 cells were removed from each flask and forced through a 21g needle about 5-10 times to break up clumps. This procedure caused the cells to lose their neurites. The cells were diluted with media to approximately  $2 \times 10^4$  cells/ml, 50  $\mu$ l were added to each well of the collagen-coated plates (1000 cells/well), and incubated for 1-2 hours to allow the cells to attach.

10        Samples to be tested were filter sterilized before use. To generate a dose-response curve, the samples were serially diluted in two-fold increments in growth medium and 50  $\mu$ l samples were added to the wells. Purified mouse NGF was serially diluted and plated in the same manner to 15 generate a standard curve. After 5 days exposure to the NGF-containing samples, plates were scored for the presence or absence of NGF activity by counting the total number of cells and the number of cells sprouting at least one neurite that is longer than twice the diameter of the 20 cell body in two or three representative fields of view. The results were expressed as the percent of cells extending neurites as a function of NGF concentration (Figure 11). Panel A is the standard curve for purified mouse NGF. Panel B is the activity curve for the NHT 25 fusion protein. The results demonstrated that the fusion protein fully retained NGF biological activity in vitro.

**EXAMPLE 12 - Construction of Other NGF-Transferrin Fusion Genes**

30        Three additional fusions of NGF and transferrin were created and tested: 1) a direct fusion of the pre-pro NGF gene and transferrin cDNA, 2) a fusion of the pre-pro NGF gene and transferrin cDNA separated by a sequence encoding five glycine residues (NGF-(gly)<sub>5</sub>-transferrin), and 3) a fusion of the pre-pro NGF gene and transferrin cDNA

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separated by a sequence encoding leu-glu (NGF-leu-glu-transferrin). The NGF-IgG3 hinge-transferrin containing vector, CD5KNHT, was employed as the source of genetic material for creating these fusions.

5 Two-step PCR reactions were performed on CD5KNHT to generate the NGF-transferrin direct fusion and the NGF-(gly)<sub>5</sub>-transferrin fusion. For NGF-leu glu-transferrin, a one-step PCR reaction was sufficient to generate the fragment of interest. Methods for constructing each of 10 these gene fusions are described below.

Construction of the NGF-Tf Direct Fusion

The following PCR primers were used:

P202 5'-AAGGAGGTGATGGTGTTGGGA-3'  
(SEQ.ID.NO.9)

15 P205 5' end of transferrin/3' end of NGF  
5'-CTCACAGTTTATCAGGGAC TCTCACAGCCTTCCTG  
CTGAGC-3'  
SEQ.ID.NO.10)

20 P204 3' end of NGF/5' end of transferrin  
5'-CAGCAGGAAGGCTGTGAGA GTCCCTGATAAAACTGT  
GAGATG-3'  
(SEQ.ID.NO.11)

P203 5'-GTGTGGCAGGACTTCTTGCCT-3'  
(SEQ.ID.NO.12)

25 P202 and P205 were primers used to amplify the 3' two-thirds of the NGF gene. P202 was complementary to a sequence within the NGF gene 5' to the unique ScaI site. P205 was complementary to 22 bases at the 3' end of the NGF gene, and 20 bases at the 5' end of the transferrin 30 gene.

Primers P204 and P203 were used to amplify the transferrin gene. P204 was complementary to a 23 base sequence at the 5' end of the transferrin gene and 19 bases at the 3' end of the NGF gene. P203 was

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complementary to a sequence within the transferrin coding sequence 3' to a unique BamHI site.

pcDNA1/AmpNHT DNA was mixed separately with the two sets of primers. As a result of PCR, two fragments were 5 amplified having overlapping protrusions creating the junction between the NGF and transferrin coding sequences. The two fragments were gel purified and an equimolar amount of each fragment was combined for a second PCR 10 amplification with primers P202 and P203. The resulting product was digested with ScaI and BamHI and exchanged with the comparable ScaI to BamHI fragment of CD5KNHT DNA that included the 3' end of the NGF gene, the hinge region and the 5' end of transferrin coding sequence. (The BamHI digestion of CD5KNHT was a partial digestion due to the 15 presence of a second BamHI site downstream of the transferrin gene.) The resulting plasmid, CD5KNT, contained a direct fusion of the NGF and transferrin genes.

Construction of the NGF-(Gly)<sub>5</sub>-Transferrin Fusion

20 The following PCR primers were used: P202 (SEQ.ID. NO. 9), P200, P201 and P203 (SEQ.ID.NO.12).

25 P200 3' end of NGF/ Gly<sub>5</sub> /  
5'-CAGCACAGGAAGGCTGTGAGA GGGGGAGGTGGAGGG  
5' end of transferrin  
GTCCCTGATAAAACTGTGAGATG-3'  
(SEQ.ID.NO.13)

30 P201 5' end of transferrin/ Gly<sub>5</sub> /  
5'-CTCACAGTTTATCAGGGAC CCCTCCACCTCCCC T  
3' end of NGF  
CTCACAGCCTTC-3'  
(SEQ.ID.NO.14)

Primer P200 was in part complementary to the 3' end of the NGF gene and contained a non-complementary region encoding five glycine residues followed by 23 bases 35 complementary to the 5' end of the transferrin coding

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sequence. Primer P201 was complementary to the 5' end of the transferrin, preceded by a non-complementary region encoding five glycine residues and the 3' end of the NGF gene.

5 pcDNA1/AmpNHT DNA was mixed separately with the two sets of primers (P202 and P201 were used to amplify the 3' two-thirds of the NGF gene and P200 and P203 were used to amplify the 5' end of the transferrin gene). Two fragments were generated having overlapping protrusions.

10 The two fragments were gel purified and an equimolar amount of each fragment was combined for a second PCR amplification with primers P202 and P203. The resulting amplified product was digested with ScaI and BamHI and exchanged for the comparable ScaI to BamHI fragment of 15 CD5KNHT DNA as described above. The resulting plasmid, CD5KNGT, contained a fusion of the NGF and transferrin coding sequences separated by a sequence encoding five glycine residues.

Construction of the NGF-leu glu-Transferrin Fusion

20 The following primers were used: P206 and P203 (SEQ. ID.NO.12).

XhoI /5' end of transferrin  
P206 5'-GGAACGGC CTCGAG GTCCCTGATAAACTGTGAGA-3'  
(SEQ.ID.NO.15)

25 Primer P206 contained an XhoI site (CTCGAG which encodes Leu-Glu) followed immediately by a 21 base sequence complementary to the 5' end of transferrin coding sequence. Primer P203 is complementary to the region 3' to the BamHI site within the transferrin gene.

30 pcDNA1/AmpNHT DNA was combined with the two primers for PCR amplification. The resulting fragment was digested with Xhol and BamHI and ligated with CD5KNHT that had been

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digested to completion with *Xba*I and partially with *Bam*HI, resulting in the deletion of the IgG3 hinge region and inclusion of a leu-glu coding sequence. The resulting plasmid, CD5KNXT, contained a fusion of the NGF and 5 transferrin coding sequence separated by a sequence encoding leu-glu.

The NGF-transferrin direct fusion, NGF-(Gly)<sub>5</sub>-transferrin fusion, and NGF-leu glu-transferrin fusion were each transfected and transiently expressed in COS 10 cells as in Example 11. Each of the expressed fusion proteins were detected by using anti-NGF antibodies, anti-transferrin antibodies or purified transferrin receptors. This demonstrates that these fusion proteins have NGF and transferrin binding characteristics similar to that of the 15 native unfused proteins.

These three fusion proteins were also subjected to the PC12 neurite outgrowth bioassay for NGF activity as described in Example 11. Each of the three fusion proteins exhibited NGF biological activity as exemplified 20 by their ability to stimulate neurite outgrowth.

### EXAMPLE 13 - Construction of a CNTF-Transferrin Fusion Gene

## Isolation and Cloning of CNTF Gene

The human CNTF gene was cloned from genomic DNA 25 prepared from Jurkat cells using PCR techniques. To obtain the CNTF gene sequence uninterrupted by intron sequences, PCR was performed in two steps using the following four primers.

30	<b>P31.1</b> 5'-AGTTAA CATATG GCTTTACTGAGCATTAC-3' (SEQ.ID.NO.16)	<b>P42.1</b> 5'-CAGGCCCTGATGCTTCACATAGGATTCCGTAAGAGC AGT-3' (SEQ.ID.NO.17)
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P35.1 5'-TACGGAAATCCTATGTGAAGCATCAGGGCCTGA ACA-3'  
(SEQ.ID.NO.18)

5 P42.2 Xhol  
5'-GGGCC CTCGAG GGACTAACTGCTACATTTCTTGTGTT  
GTT AGC-3'  
(SEQ.ID.NO.19)

P31.1 and P42.1 were the 5' and 3' primers for amplifying the 5' exon of CNTF. P31.1 was complementary to the 5' end of the 5' exon and contained an NdeI cloning site. P42.1 contained 21 bases complementary to the 3' end of the 5' exon and 18 bases complementary to the 5' end of the 3' exon.

P35.1 and P42.2 were the 5' and 3' primers for amplification of the 3' exon of CNTF. P35.1 was complementary to the 3' exon and contained 13 additional bases which were complementary to the 3' end of the 5' exon. P42.2 was complementary to the 3' end of the 3' exon and contained an XhoI cloning site.

Genomic DNA was mixed separately with the two sets of 20 primers. A 120 bp fragment was amplified with primers P31.1 and P42.1, and a 480 bp fragment was amplified with primers P35.1 and P42.2. The two fragments had a total of 31 base pairs of overlapping sequence.

In the second PCR reaction, a 600 bp fragment was 25 amplified using a mixture of gel purified 120 bp and 480 bp fragments as templates together with primers P31.1 and P42.2 (described above). The amplified fragment was digested with NdeI and XhoI and gel purified.

The NdeI-XhoI fragment was cloned into *E. coli* 30 expression vector pET17xb (Novagen) that had been gel isolated after digestion with NdeI and XhoI. The ligated product was transformed into competent *E. coli* MC1061 cells. A clone containing the CNTF gene fragment was identified by restriction digestion and DNA sequence 35 analysis and designated J6.

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Cloning of Human CNTF Coding Sequence into a Mammalian Expression Vector

The human CNTF coding sequence was cloned into the CD51neg1 mammalian expression vector of Example 12. By 5 the methods described below, the CNTF gene was cloned into the CD51neg1 expression vector, adjacent to the CD5 leader sequence, in place of the IgG1 sequences.

To prepare CNTF DNA having terminal restriction sites compatible with sites in CD51neg1, a DNA fragment was 10 generated by PCR techniques using clone J6 as a template and P33.1 and P35.2 as 5' and 3' PCR primers, respectively. P33.1 was complementary to the 5' end of the CNTF gene and contained a BlnI restriction site rather than the NdeI site. P35.2 was, in part, complementary to 15 the 3' end of the CNTF gene and contained an EagI restriction site rather than the XbaI site.

20 BlnI  
P33.1 5'-CGCGGG CCTAGG CGCTTTCACAGAGCATTCAAC-3'  
(SEQ. ID. NO. 20)

EagI  
P35.2 5' CGCGGGG CGGGCG CTTTACATTTCTTGTGTTGTTAG-3'  
(SEQ. ID. NO. 21)

Following PCR, the resulting amplified DNA fragment was treated with BlnI and EagI and ligated with the NheI and 25 EagI digested CD51neg1 vector backbone (NheI and BlnI generate comparable protrusions; neither site is regenerated after ligation). The ligated mixture was transformed into competent *E. coli* MC1061 cells. A resulting clone having the CNTF gene preceded by the CMV 30 promoter and CD5 leader was identified by restriction digestion and designated D1.

In order to minimize the possibility that the CNTF gene present in plasmid D1 contained mutations resulting

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from PCR procedures, NheI and BamHI, each of which cleaves once within CNTF, were used to isolate a fragment containing most of the CNTF coding sequence from D1 and exchanged for the equivalent NheI-BamHI fragment of CNTF 5 from J6. The resulting plasmid was designated d1 (see Figure 12A).

#### Construction of CNTF-Transferrin Gene Fusions

Three different gene fusions encoding CNTF-transferrin fusion proteins were constructed. In each 10 case, the proteins were connected by different linking sequences. In the first of these constructs, CNTF and transferrin were connected by the hinge region of IgG3 (CNTF-IgG3 hinge-transferrin). In the second construct, the CNTF and the transferrin sequences were joined without 15 an intervening linker (CNTF-transferrin). In the third construct, a sequence coding for penta-glycine was inserted between the CNTF and transferrin genes (CNTF-(Gly)<sub>5</sub>-transferrin). Methods for constructing each of these gene fusions are described below.

##### 20 A. CNTF-IgG3 Hinge-Transferrin

The gene fusion coding for CNTF-IgG3 hinge-transferrin was constructed for expression in the CD51neg1 expression vector using the following multi-step procedure. First, the transferrin gene was isolated and 25 cloned into CD51neg1 in place of the IgG1 sequences. To do this, a fragment containing the transferrin gene was generated by PCR techniques using pcDNA1/AmpNHT of Example 11 as the template and oligonucleotides P33.2 and P36.1 as 3' and 5' PCR primers, respectively. P33.2 was 30 complementary to the 5' end of the transferrin gene and contained a BlnI site. P36.1 was complementary to the 3' end of the gene and contained an EagI site.

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P33.2 BlnI 5'-GCTTCCGT CCTAGG GGTCCCTGATAAAACTGTG-3' (SEQ. ID. NO. 22)

5 P36.1 5-CGCGGG CGGCG CTTTAAGGTCTACGGAAAGTGCA-3' (SEQ. ID. NO. 23)

Following PCR, the amplified fragment was digested with BlnI and EagI and cloned into the NheI and EagI digested and gel purified CD5Inegl backbone. The 10 resulting plasmid, having the transferrin gene inserted adjacent to and downstream of the CD5 leader in place of the IgG1 hinge-Fc, was designated C4. As before, in order to eliminate the possibility that errors may have been incorporated during PCR amplification, a BamHI to Asp718 15 fragment of C4, which contained most of the transferrin gene sequence, was replaced with the equivalent BamHI-Asp718 fragment of the starting plasmid pcDNA1/AmpNHT. The resulting plasmid was designated C\*. (See Figure 12B).

20 The IgG3 hinge region sequence was then inserted upstream of the transferrin coding sequence in C4 as follows. The Xhol-Asp718 fragment containing the IgG3 hinge and the 5' portion of the transferrin coding sequence (with the ala-ala coding sequence immediately 25 upstream of the transferrin coding sequence) from pcDNA1/AmpNHT was isolated and cloned into the Xhol and Asp718 digested C4 backbone to generate C4-NHT. (See Figure 12C).

Next, a gene fusion consisting of the CNTF gene and the transferrin gene was created in the CD51neg1 backbone. First, a 0.7 kb *Xba*I-*Bam*HI fragment from clone d1 (containing the CD5 leader and most of the CNTF gene except for the 3' end) and a 1.8 kb *Bam*HI-*Eag*I fragment from C4-NHT (containing most of the transferrin coding

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sequences except for the 5' end) were ligated with *Xba*I and *Eag*I digested and gel-purified CD51neg1 backbone to generate  $q^*$ . (See Figure 12n)

Then, a fragment containing the IgG3 hinge region was inserted between the CNTF and transferrin genes. In order to accomplish this, an *Xba*I site was created at the 3' end of the CNTF gene to make it compatible with the *Xba*I site at the 5' end of the hinge region sequence. (This *Xba*I site encoded a leu-glu 5' to the IgG3 hinge region). A new CNTF containing fragment was generated by PCR techniques using J6 as the template and primers P33.1 (above) and P36.5 (containing an *Xba*I site).

15 P36.5 5' TGGCCTCTCACC CTCGAG CATTTCCTTGTGTT  
AGC-3'  
(SEQ. ID. NO. 24)

The PCR product was digested with BlnI and XhoI and cloned into SpeI (compatible with BlnI) and XhoI digested C4-NHT to generate H45. H45 contained the CNTF gene joined to 20 the hinge coding sequence which was in turn joined to the transferrin gene. (See Figure 12E).

Finally, a 1.5 kb BamHI fragment from H45, which contained the 3' end of the CNTF gene, the hinge coding sequence and the 5' end of the transferrin gene, was isolated and cloned into BamHI digested g\* to generate plasmid gH, a plasmid containing the CNTF-hinge-transferrin gene fusion downstream of the CMV promoter and the CD5 leader sequence. (See Figure 12F).

#### B. CNTF-Transferrin Direct Fusion

30 The CNTF-transferrin direct fusion was made by a two  
step PCR procedure. In the first step, with J6 as the  
template, the CNTF coding sequence was amplified using  
primers P33.1 (SEQ.ID.NO.20, described above) and P38.1  
(below), and the transferrin gene was amplified using

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primers P36.2 (below), P36.1 (SEQ. ID. NO. 23 described above) and template pcDNA1/AmpNHT. P38.1 was complementary to the 5' end of the transferrin gene and the 3' end of the CNTF coding sequence. P36.2 was 5 complementary to the 3' end of the CNTF coding sequence and the 5' end of the transferrin gene.

P38.1

10 5' end of transferrin/3' end of transferrin  
5'-CTCACAGTTTATCAGGGAC CATTTCCTTGTTAGC-3'  
(SEQ.ID.NO.25)

P36.2

3' end of CNTF / 5' end of transferrin  
5'-GCTAACAAACAGAAAATG GTCCCTGATAAAACTGTG-3'  
(SEQ.ID.NO.26)

15 The two amplified fragments were gel purified and equimolar amounts were combined for the second PCR using primers P33.1 and P36.1. The product, an intact fragment containing a CNTF-transferrin fusion gene, was digested with BlnI and EagI and ligated with NheI and EagI digested 20 CD51neg1. A clone was identified by restriction analysis and designated A45. In order to minimize the possibility of mutations introduced by PCR, a BamHI fragment from A45, which spanned the joint between the CNTF and transferrin gene sequences, was cloned into BamHI digested g\*. The 25 resulting plasmid was designated gA.

#### C. CNTF-(Gly)<sub>5</sub>-Transferrin Fusion

The CNTF-transferrin fusion separated by a series of nucleotides which encode five glycine residues was similarly constructed using two primer pairs and templates 30 J6 and pcDNA1/AmpNHT. Primers P33.1 (SEQ. ID. NO. 20) and P53.2 (below) were complementary to the 5' and 3' ends of the CNTF gene. In addition, P53.2 contained an

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intervening nucleotide sequence encoding five glycines. Primers P53.1 (below) and P36.1 (SEQ. ID. NO. 23) were complementary to the 5' and 3' ends of the transferrin gene. P53.1 also contained the nucleotide sequence 5 encoding five glycines and this sequence overlapped with the 3' ends of the fragment generated with primers P33.1 and P53.2.

P53.2

10 5' end of transferrin / Gly<sub>5</sub>  
5'-CTCACAGTTATCAGGGAC CCCTCCACCTCC  
/ 3' end of CNTF  
CCC CATTCTCTGTTAGC-3'  
(SEQ.ID.NO.27)

P53.1

15 3' end of CNTF/ Gly<sub>5</sub> /  
5'-GCTAACACAAGAAAATG GGGGGAGGTGGAGGG GT  
5' end of transferrin  
CCCTGATAAAACTGTGAG-3'  
(SEQ.ID.NO.28)

20 After the first PCR reaction, the two amplified fragments were purified, annealed and subjected to a second round of PCR using primers P33.1 and P36.1. The final product was digested with BlnI and EagI and ligated with NheI and EagI digested CD51negI DNA. A clone was 25 identified by restriction analysis and designated B45. The BamHI fragment was again exchanged for the BamHI fragment in g\*, resulting in a plasmid designated gB.

30 Competition assays for transferrin receptor binding activity were performed as described in Example 11 to measure the affinity of the CNTF-transferrin fusion proteins for the human transferrin receptor. The results of these assays demonstrated that the CNTF-transferrin fusion proteins bind well to the human transferrin receptor.

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A cell-based bioassay described by Collins *et al.* (1989, *Brain Res.* **502**: pp. 99-108) was used to assay CNTF activity in the CNTF-transferrin fusions by measuring the ability of these fusion proteins to stimulate outgrowth of 5 isolated neurons. The results of these bioassays demonstrated that all fusion proteins retained CNTF activity.

**EXAMPLE 14 - Construction of NGF-Anti-Transferrin Receptor Antibody Fusion Genes, Expression of NGF-Anti-Transferrin Receptor Antibody Fusion Genes as Fusion Proteins and Assay of the Fusion Protein Constituents**

Fusion proteins were constructed which were comprised of human NGF and a chimeric antibody that recognizes the human transferrin receptor. The NGF sequence was joined 15 to the N-terminus of the heavy chain of the antibody molecule through a short linker segment consisting of two alanine residues. As described below, separate expression plasmids encoding the light chain and the heavy chain of the antibody and their fusion derivatives were initially 20 constructed to facilitate screening by transient transfection. Subsequently, the heavy and light chain sequences were incorporated into a single expression plasmid.

**Preparation of Antibody Light Chain Expression Plasmids**

25 The vector pRC/CMVL was used for the construction of antibody light chain mammalian expression plasmids. This vector is a derivative of the pRC/CMV plasmid (Invitrogen) which has been modified to carry a larger version of the cytomegalovirus (CMV) major immediate early (MIE) 30 promoter, by inserting a MluI-HindIII fragment derived from pEE14 (Celltech, Ltd.) into the MluI-HindIII digested pRC/CMV plasmid.

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The initial construct, pCMV $\kappa$ 12, contained the light chain variable region of the murine 128.1 anti-human transferrin receptor monoclonal antibody (see Example 10) fused directly to a human kappa light chain constant 5 domain. One way to construct this plasmid is to ligate together a HindIII-PpuMI fragment derived from the plasmid pAG4611 (WO 93/10819 published 10 June 1993; pertinent portions of which are herein incorporated by reference) containing the sequence coding for the 128.1 variable 10 region, along with two adjacent fragments encoding the human kappa constant region, into HindIII and XbaI cleaved pRC/CMVL. The two fragments encoding the human kappa constant region are derived from pAG4611, but are modified to contain an XbaI site immediately following the 3' end 15 of the kappa coding region. Introduction of the XbaI site may be performed in the following manner.

A PCR primer pair consisting of one primer that is complementary to the 5' end of the kappa sequence just upstream of an XmnI site, and a second primer that is 20 complementary to the 3' end of the kappa sequence and contains an XbaI restriction site, is used to generate a fragment of the human kappa constant region.

Primer A 5'-CTGTTGTGTGCCTGCTGAATA-3'  
(SEQ.ID.NO.29)

25 Primer B 5'-GCGTACGTACG TCTAGA AAC TAA CACTCATT  
XbaI stop  
CCTGTTGAA-3'  
(SEQ.ID.NO.30)

The PCR generated fragment amplified off the pAG4611 30 template is digested with XmnI and XbaI and ligated along with a PpuMI-XmnI fragment from pAG4611 (containing the 5' end of the kappa constant region coding sequence) and a HindIII-PpuMI fragment from pAG4611 into HindIII and XbaI cleaved pRC/CMVL creating pCMV $\kappa$ 12. This plasmid was used

to generate pCMV $\kappa$ IVS21, which restored the intron present between the variable and constant domain coding exons. This plasmid was constructed by exchanging a 0.4 kb PpuMI-XmnI fragment from pCMV $\kappa$ 12 with the intron-containing 3.0 5 kb PpuMI-XmnI fragment derived from pAG4611.

Preparation of Antibody Heavy Chain and NGF-Heavy Chain Fusion Expression Plasmids

The chimeric antibody sequences used in the construction of the heavy chain expression plasmids were 10 derived from the plasmid pAH4602 (WO 93/10819 published 10 June 1993; pertinent portions of which are herein incorporated by reference) and consisted of the heavy 15 chain variable region of the murine 128.1 anti-human transferrin receptor monoclonal antibody fused to human genomic DNA encoding the IgG1 constant region. A 3.0 kb EcoRV-BamHI fragment of pAH4602 carrying the complete chimeric heavy chain gene was cloned into the mammalian expression vector pEE13 (Celltech, Ltd.) between the SmaI and BclI sites, creating the plasmid pEE $\gamma$ 1. This plasmid 20 was then modified to contain a unique HindIII site immediately following the promoter by eliminating a second HindIII site downstream of the heavy chain coding region. The second HindIII site was eliminated by using DNA 25 polymerase to fill in a HindIII partially digested plasmid followed by religation. This pEE $\gamma$ 1 derivative was designated pEE $\gamma$ HIIIb.

The plasmid which expresses human NGF joined to the amino terminus of the antibody heavy chain was constructed using the following multi-step procedure. The 5' 30 untranslated region and the segment which encodes the signal sequence of the chimeric 128.1 heavy chain were deleted from the pEE $\gamma$ 1HIIIb plasmid and replaced with a NotI site-containing linker segment. This linker segment encodes a pair of alanine residues in frame with the

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mature amino terminus of the heavy chain variable region. This replacement was done using PCR-mediated mutagenesis to generate the modified DNA fragment which was then exchanged into the pEE $\gamma$ 1HIIb plasmid between the HindIII 5 and NheI sites. Mutagenesis was carried out using pAH4602 as the template and oligonucleotides #375 and #376 as the 5' and 3' PCR primers, respectively. Primer #375 was complementary to the 5' end of the 128.1 heavy chain gene with the addition upstream of a HindIII site followed by 10 the NotI (Ala-Ala) linker. Primer #376 was complementary to a segment found in the CH1-encoding portion of the antibody gene.

#375	<pre>       HindIII      NotI       5' -GGGG AAGCTT TT GCGGCCGC TGAGGTCCAGCTGCAAC       AGTCTG-3'       (SEQ. ID. NO.31)     </pre>
15	
#376	<pre>       5' -CCGTCTGGTCAGGGCGCCTGAGTT-3'       (SEQ. ID. NO.32)     </pre>

The amplified PCR fragment was digested with HindIII and NheI and then ligated with the HindIII-NheI vector fragment isolated from pEE $\gamma$ 1HIIb. Following transformation into the E.coli strain XL-1 Blue, a clone containing the modified 128.1 heavy chain gene was identified by restriction digestion and verified by DNA sequencing. This plasmid, pEE $\gamma$ 1HIIb/NotI, served as an intermediate in the construction of the final NGF-antibody fusion plasmid.

A second intermediate construct, pRC/CMVLF-NFG120, containing the human preproNGF gene in which the 3' coding sequence was altered to introduce a NotI site was generated in the following manner. In the plasmid pRC/CMVLF-NFG120, the preproNGF is preceded by a Kozak sequence (described in Example 11) and is under the control of the long version of the CMV MIE promoter (described above). One way to construct pRC/CMVLF-NFG120

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is to first remove the HindIII-SmaI insert from pGEM2/KNGF (Example 11) and insert it into HindIII and SmaI digested pEE14 (Celltech, Ltd.) creating pEENGF7. An approximately 1000 base pair fragment containing the Kozak sequence-5 prepro NGF gene and an SV40 poly A site (present in the pEE14 vector) is removed from pEENGF7 by complete digestion with HindIII and partial digestion with BamHI. This fragment is then inserted into HindIII and BamHI digested pRC/CMVL to create pRC/CMVL-NGF120.

10 In order to facilitate fusion to the antibody heavy chain, pRC/CMVL-NGF120 was then modified by introducing a NotI site at the 3' end of the coding sequence in the following manner. The PCR primer pair #219/#382 was used to generate an altered DNA fragment incorporating these 15 modifications. Forward (5') primer #219 is identical to a segment within the human NGF prepro-coding sequence, while reverse (3') primer #382 is complementary to the 3' end of the NGF coding sequence and carries an additional NotI restriction site which encodes an Ala-Ala dipeptide in- 20 frame with the C-terminus of NGF.

#219 5'-GCGCCCCGGCAGCGCGATAG-3'  
(SEQ.ID.NO.33)

25 #382 XbaI NotI 5'-GGGGG TCTAGA CGGGCCGC TCTTCACAGCCTTC  
CTGCTG-3'  
(SEQ.ID.NO.34)

The PCR fragment amplified off the pRC/CMVL-NGF120 template was cleaved with EcoRI and XbaI and ligated back into the EcoRI/XbaI cut plasmid. Following transformation 30 into E.coli strain XL-1 Blue, a clone containing the modified NGF plasmid was identified by restriction digestion and verified by DNA sequencing. From this plasmid, named pRC/CMVL-NGFNotI, a HindIII-NotI fragment carrying the entire modified preproNGF gene was obtained

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and ligated into the HindIII and NotI digested pEE $\gamma$ 1HIIIB/NotI heavy chain plasmid. The product derived from this ligation, plasmid pRC/CMVL $\gamma$ 1NGF-4, encoded the complete proproNGF-(Ala)<sub>2</sub>-128.1 heavy chain fusion protein.

Construction of the Combined 128.1 Light Chain/NGF-128.1 Heavy Chain Expression Plasmid

Following functional assessment in transient transfection assays using COS7 cells, the entire light chain transcription unit was excised from the pRC/CMVL vector backbone and transferred into the NGF-heavy chain expression plasmid. To accomplish this, the light chain expression plasmid pCMV $\kappa$ IVS21 was cleaved with BamHI and partially digested with BglII to isolate a 6.4 kb fragment containing the CMV promoter, the light chain gene, and the polyadenylation/termination signals. This DNA segment was ligated into pRC/CMVL $\gamma$ 1NGF-4 which had been cleaved with BamHI and treated with alkaline phosphatase. After transformation into *E.coli* and screening by PCR and restriction digestion, plasmids were identified which contained the light chain unit inserted in either of the two possible orientations into the BamHI site. These two plasmid constructs were referred to as pEEAK-30 $\kappa$ / $\gamma$ 1NGF5-4 with both the heavy and light chain gene transcription units in the same orientation and pEEAK-30 $\gamma$ 1NGF/ $\kappa$ 5-12 where the heavy and light chain genes are convergently transcribed.

Assay for NGF in the NGF-Antibody Fusion Protein Expressed in Mammalian Cells.

30 Expression plasmid pEEAK-30 $\gamma$ 1NGF/ $\kappa$ 5-12 was transfected and transiently expressed in COS cells. The NGF-anti-transferrin receptor antibody fusion protein

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(designated NAK) in culture supernatants was detected by standard ELISA procedures.

Briefly, capture antibody (anti-human IgG, Vector Labs), which was specific for human IgG1 was coated in the 5 wells of a 96-well plate. The wells were washed (PBS-0.05% Tween), blocked with 1% bovine serum albumin (BSA), and supernatants from transfected COS cells were added to the wells (typically in serial four-fold dilutions) and incubated for one hour at room temperature.

10 An anti-NGF detection antibody (rat monoclonal antibody 1G3) was chosen which would recognize the NGF portion of the NAK fusion protein and was added to the wells on top of the fusion protein. Bound antibody was detected by peroxidase reaction after amplification of the 15 signals by an avidin-biotin reaction using biotinylated anti-rat antibody (VectorLabs) and the Vectastain ABC kit (VectorLabs). Protein was quantitated by extrapolation from standard curves generated for known concentrations of NGF.

20 Alternative ELISA procedures may be used to detect and quantitate the fusion proteins. For example, the capture antibody and detection antibody may recognize the same portion of the fusion protein.

To produce stable cell lines expressing NAK, 25 linearized vector pEEAK-30 $\gamma$ 1NGF/ $\kappa$ 5-12 was electroporated into CHO cells and the cells were subjected to drug selection in 25 mM methionine sulfoximine (MSX). Expression of the NAK fusion protein was assayed by ELISA as described above. Proteins containing both the NGF and 30 anti-transferrin receptor antibody sequences were expressed and detected in the culture supernatant.

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Purification of NGF-Anti-Transferrin Receptor Antibody Fusion Protein from COS Cell Supernatants.

NGF-anti-transferrin receptor antibody fusion protein present in the supernatant of transfected COS cells was 5 purified by using a Protein A affinity column (Perseptive Biosystems, Cambridge, MA) followed by a Poros HS/50 cation exchange column (Perseptive Biosystems) according to the manufacturer's instructions.

The affinity purified fractions contained two major 10 bands at 63kD and 24kD MW on a reducing SDS-polyacrylamide gel. Both bands were recognized by anti-human IgG1 antibody. The 63kD band was also recognized by an anti-NGF antibody.

In vitro Competition Assay for Transferrin Receptor 15 Binding Activity

A critical attribute of the NAK fusion protein is its ability to bind to the transferrin receptor. Assays were performed to measure the affinity of the NAK fusion protein for the human transferrin receptor by the ability 20 of the fusion protein to compete with native anti-transferrin receptor antibody (128.1) for binding to the transferrin receptor.

Competition assays were performed as in Example 11 with the exceptions that soluble human transferrin 25 receptor was used and a mixture of 5 mM horseradish peroxidase labeled anti-transferrin receptor antibody and varying concentrations of NAK fusion protein were applied to each well. The results of these competition assays demonstrated that the NAK fusion protein binds to the 30 human transferrin receptor with a slightly reduced affinity compared to the anti-transferrin receptor antibody.

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In vitro Assay for NGF Activity.

A cell-based bioassay using cell line 6-24 (obtained from David Kaplan, Frederick Cancer Research Center, Frederick, MD) was used to assess whether the expressed 5 NAK fusion protein has NGF activity by measuring its ability to stimulate neurite outgrowth from these cells. The 6-24 cell line is derived from PC12 cells that have been engineered to overexpress the trkA high affinity NGF receptor by transfecting the PC12 cells with a trkA 10 expression vector.

The 6-24 cells were grown in DMEM medium (Bio Whittaker) containing 5% fetal calf serum, 10% horse serum and 2 mM L-glutamine, in T75 flasks under 5% CO<sub>2</sub>. Ninety-six well plates were coated with 1.0 µg/cm<sup>2</sup> bovine 15 collagen IV in 0.05N HCl at 50 µl/well for 1 hour at room temperature and washed 3 times with PBS. Five ml of 6-24 cells were removed from each flask and forced through a 21g needle about 2-5 times to break up clumps. This procedure caused the cells to lose their neurites. The 20 cells were diluted with media to approximately 2x10<sup>4</sup> cells/ml, 50 µl were added to each well of the collagen-coated plates (1000 cells/well), and incubated overnight at 37 °C to allow the cells to attach.

Samples to be tested were filter sterilized before 25 use. To generate a dose-response curve, the samples were serially diluted in two-fold increments in growth medium and 50 µl samples were added to the wells. Purified mouse NGF was serially diluted and plated in the same manner to generate a standard curve. After 1 day exposure to the 30 NGF-containing samples, plates were scored for the presence or absence of NGF activity by counting the total number of cells and the number of cells sprouting at least one neurite that is longer than twice the diameter of the cell body in two or three representative fields of view. 35 The results were expressed as the percent of cells

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extending neurites as a function of NGF concentration. The results of this assay demonstrated that the fusion protein fully retained NGF biological activity in vitro.

Equivalents

5 Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments expressly described herein. These are intended to be within the scope of the invention as described by the claims herein.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Alkermes, Inc.  
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(D) STATE: Ca  
(E) COUNTRY: 94612  
(F) POSTAL CODE (ZIP): USA

(ii) TITLE OF INVENTION: Transferrin Receptor Specific  
Ligand-Neuropharmaceutical Agent Fusion Proteins

(iii) NUMBER OF SEQUENCES: 34

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/094,534  
(B) FILING DATE: 16-JUL-1993

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/999,803  
(B) FILING DATE: 20-NOV-1992

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/846,830  
(B) FILING DATE: 06-MAR-1992

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(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 07/404,089
- (B) FILING DATE: 07-SEP-1989

(1) INFORMATION FOR SEQ ID NO: 1

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGGTCGACCT CGAGGGTGAG AGGCCAGC

28

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGAGTTACTC AGATCTGGGA AG

22

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GAAGCTTCTC GAGTCTAGAC CAGGTGCATA GCGTAATGTC C

41

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CGTCGACCTC GAGTCTCACA GCCTTCCTGC TGAGC

35

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CGTCGACTCT AGATTATCTC ACAGCCCTTC

29

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- 68 -

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCCCCCGGGT CTAGACCAGG TGCATCCACC ATGGTCCATGT TGTTC

45

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AACAGCTATG ACCATG

16

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1459 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(B) STRAIN: CD51neg1

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: CD5 Leader; IgG1 Exon 1; IgG1 Exon

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2; IgG1 Exon 3  
 (B) MAP POSITION: 97-177; 535-593; 698-1027; 1124-1444

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CTCGAGATCC ATTGTGCTCT AAAGGAGATA CCCGGCCAGA CACCCCTCACC TCGGGTCCCC	60
AGCTGCCAG GCTGAGGCCA GAGRAGGCCA GAAACCATGC CCATGGGTC TCTGCACCCG	120
CTGGCCACCT TGTACCTGCTG GGGGATGCTG GTCGCTTCGG TGTAGCGGA TCCCGAGGGT	180
GAGTACTAAG CTTCAAGCGCT CCTGCCTGGA CGCATCCCGG CTATGCAGCC CCAGTCCAGG	240
GCAGCAGGGC AGGGCCCGTC TGCCCTCTCA CCCGGAGCCT CTGCCCCGCC CACTCATGCT	300
CAGGGAGAGG GTCTTCTGGC TTTCCTCCAG GCTCTGGCA GGCACAGGCT AGGTGCCCCCT	360
AACCCAGGGC CTGCCCCACAA AGGGCAGGT GCTGGGCTCA GACCTGCCAA GAGCCATATC	420
CGGGAGGACC CTGCCCCCTGA CCTAAAGGCCA CCCAAAGGC CAAACTCTCC ACTCCCTCAG	480
CTCGGACACC TTCTCTCTTC CCAGATTCCA GTAACTCCCA ATCTTCTCTC TGCAGAGGCC.	540
AAATCTTGTG ACAAAACTCA CACATGCCCA CGGTGCCAG GTAGGCCAGC CCAGGCCCTG	600
CCCTCCAGCT CAAGGCGGGA CAGGTGCCCT AGAGTAGCCT GCATCCAGGG ACAGGGCCCA	660
GCGGGGTGCT GACACGTCCA CCTCCATCTC TTCTCTGGCA CCTGAACTCC TGGGGGGACC	720
GTCAGTCTTC CTCTTCCCCA CAAACCCCA GGACACCTCTC ATGATCTCCC GGACCCCTGA	780
GGTCACATGC GTGGTGGTGG ACGTGAGCCA CGRAGACCCCT GAGGTCAAGT TCAACTGGTA	840
CGTGGACGGC GTGGAGGTGC ATAATGCCAA GACAAAGCCG CGGGAGGAGC AGTACAAACAG	900
CACGTACCGG GTGGTCAGCG TCCTCACCGT CCTGCACCCAG GACTGGCTGA ATGGCAAGGA	960
GTACAAGTGC AAGGTCTCA ACAAAAGCCCT CCCAGCCCCC ATCGAGAAAA CCTCTCCAA	1020
AGCCAAAGGT GGGACCCGTG GGGTGGCGAGG GCCACATGGA CAGAGGCCGG CTGGCCAC	1080

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CCTCTGCCCT GAGAGTGACC GCTGTACCAA CCTCTGTCCT ACAGGGCAGC CCGGAGAAC	1140
ACAGGGTGTAC ACCCTGCCCC CATCCCGGGA TGAGCTGACC AAGAACCCAGG TCAGCCTGAC	1200
CTGCGCTGGTC AAAGGCTTCT ATCCCAGCGA CATCGCCGTG GAGTGGGAGA GCAATGGGCA	1260
GCCGGAGAAC AACTACAAGA CCACGCCTCC CGTGCTGGAC TCCGACGGCT CCTTCTTCCT	1320
CTACAGCAAG CTCACCGTGG ACAGAGGAG GTGGCAGCAG GGGAAACGTCT TCTCATGCTC	1380
CGTGATGCAT GAGGCTCTGC ACAACCACCA CACGCAGAAG AGCCTCTCCC TGTCTCCGGG	1440
TAAATGAGTG CGACGGCCG	1459

## (2) INFORMATION FOR SEQ ID NO: 9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AAGGAGGTGA TGGTGTGGG A

21

## (2) INFORMATION FOR SEQ ID NO: 10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

- 71 -

CTCACAGTTT TATCAGGGAC TCTCACAGCC TTCCCTGCTGA GC

42

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CAGCAGGAAAG GCTGTGAGAG TCCCTGATAA AACTGTGAGA TG

42

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GTGTGGCAGG ACTTCTTGCC T

21

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 57 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

-72-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CAGCAGGAAG GCTGTGAGAG GGGGAGGTGG AGGGGTCCCT GATAAAACTG TGAGATG

57

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CTCACAGTTT TATCAGGGAC CCCTCCACCT CCCCTCTCA CAGCCTTC

48

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GGAACGGCCT CGAGGTCCCT GATAAAACTG TGAGA

35

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid

- 73 -

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

AGTTAACATA TGGCTTTAC TGAGCATTC A C

31

(2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 39 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CAGGCCCTGA TGCCTCACAT AGGAAITCCGT AAGAGCAGT

39

(2) INFORMATION FOR SEQ. ID NO: 18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TACGGAATCC TATGTGAAGC ATCAGGGCCT GARCA

35

(2) INFORMATION FOR SEQ ID NO: 19:

-74-

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GGGCCCTCGA GGGACTAACT GCTACATTTT CTTGTTGTTA GC

42

## (2) INFORMATION FOR SEQ ID NO: 20:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CGCGGGCTTA GGCGCTTTCA CAGAGCATTC ACC

33

## (2) INFORMATION FOR SEQ ID NO: 21:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

-75-

CGCGGGGCGG CCGCTTTACA TTTTCTTGTT GTTGTGTTAG

38

(2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 33 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GCTTCCGTCC TAGGGGTCCC TGATAAAACT GTG

33

(2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 36 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CGCGGGGCGG CCGCTTTAAG GTCTACGGAA AGTGCA

36

(2) INFORMATION FOR SEQ ID NO: 24:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 36 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

-76-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

TGGCCTCTCA CCCTCGAGCA TTTTCTTGTG GTTAGC

36

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CTCACAGTTT TATCAGGGAC CATTTCCTTG TTGTTAGC

38

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GCTAACAAACA AGAAAATGGT CCCTGATAAA ACTGTG

36

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 53 base pairs
- (B) TYPE: nucleic acid

-77-

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GTCACAGTTT TATCAGGGAC CCCCTCACCT CCCCCCCATT TCTTGTGTT AGC

53

(2) INFORMATION FOR SEQ ID NO: 28:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 53 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GCTAACAAACA AGAAAATGGG GGGAGGTGGA GGGGTCCCTG ATAAAACTGT GAG

53

(2) INFORMATION FOR SEQ ID NO: 29:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CTGTTGTG TG CCTGCTGAAT A

21

(2) INFORMATION FOR SEQ ID NO: 30:

-78-

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GCGTACGTAC GTCTAGAAC TAACTCAT TCCCTGTTGAA

40

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GGGGGARGCTT TTGCGGCCGC TGAGGTCCAG CTGCAACAGT CTG

43

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

-79-

CCGCTGGTCA GGGCGCCTGA GTT

23

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

GCGCCCCGGC AGCGGGCGATA G

21

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GGGGGTCTAG AGCGGGCCGCT CTTCTCACAG CCTTCCTGCT G

41

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CLAIMS

1. A method for delivering a neuropharmaceutical agent across the blood brain barrier to the brain of a host which comprises administering to the host a fusion protein that includes a brain capillary endothelial cell receptor ligand and said neuropharmaceutical agent, whereby said administering occurs under conditions wherein binding of said ligand to its complementary brain endothelial cell receptor occurs and the neuropharmaceutical agent is transferred across the blood brain barrier in a pharmaceutically active form and in a therapeutically effective amount.
2. The method of claim 1 wherein the neuropharmaceutical agent is selected from the group consisting of a growth factor, superoxide dismutase, CD4, a lymphokine, a lymphokine antagonist, a cytokine, a cytokine antagonist, dopamine decarboxylase and tricosanthin.
3. The method of Claim 2 wherein the neuropharmaceutical agent is a growth factor selected from the group consisting of nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), glial cell-line derived neurotrophic factor (GDNF), neurotrophin 3 (NT-3), neurotrophin 4 (NT-4), neurotrophin 5 (NT-5) and fibroblast growth factor (FGF).
4. The method of Claim 3 wherein the neuropharmaceutical agent is NGF.
5. The method of Claim 3 wherein the neuropharmaceutical agent is CNTF.

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6. The method of Claim 1 wherein the ligand is selected from the group consisting of transferrin, insulin-like growth factor 1 (IGF 1), insulin-like growth factor 2 (IGF 2), insulin, an antibody to the transferrin receptor, an antibody to insulin-like growth factor 1 (IGF 1) receptor, an antibody to insulin-like growth factor 2 (IGF 2) receptor and an antibody to insulin receptor.
7. The method of Claim 1 wherein the ligand and the neuropharmaceutical agent are linked through an intermediate peptide or polypeptide.
8. The method of Claim 7 wherein the intermediate polypeptide is an amino acid sequence from an antibody chain.
9. The method of Claim 8 wherein the intermediate polypeptide is an IgG<sub>3</sub> hinge region.
10. The method of Claim 9 wherein the ligand is transferrin and the neuropharmaceutical agent is nerve growth factor (NGF).
11. The method of Claim 9 wherein the ligand is transferrin and the neuropharmaceutical agent is ciliary neurotrophic factor (CNTF).
12. The method of Claim 7 wherein the intermediate peptide is five glycines (gly)<sub>5</sub>.
13. The method of Claim 12 wherein the ligand is transferrin and the neuropharmaceutical agent is nerve growth factor (NGF).

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14. The method of Claim 7 wherein the ligand is an anti-transferrin receptor antibody and the neuropharmaceutical agent is nerve growth factor (NGF).
- 5 15. The method of Claim 14 wherein the anti-transferrin receptor antibody is a chimeric antibody.
16. The method of Claim 12 wherein the ligand is transferrin and the neuropharmaceutical agent is ciliary neurotrophic factor (CNTF).
- 10 17. The method of Claim 7 wherein the intermediate peptide is leucine-glutamic acid (leu-glu).
18. The method of Claim 17 wherein the ligand is transferrin and the neuropharmaceutical agent is nerve growth factor (NGF).
- 15 19. A method for delivering a neuropharmaceutical agent across the blood brain barrier to the brain of a host which comprises administering to the host a fusion protein-neuropharmaceutical agent conjugate under conditions whereby binding of the fusion protein to a brain capillary endothelial cell receptor occurs and the neuropharmaceutical agent is transferred across the blood brain barrier in a pharmaceutically active form and in a therapeutically effective amount, wherein the fusion protein includes a brain capillary endothelial cell receptor ligand and an antibody or fragment thereof that is reactive with a brain capillary endothelial cell receptor.
- 20
- 25

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20. The method of Claim 19 wherein the ligand and the antibody or fragment thereof are reactive with the same brain capillary endothelial cell receptor type.
- 5 21. The method of Claim 20 wherein the brain capillary endothelial cell receptor type is the transferrin receptor and said ligand is transferrin.
22. The method of Claim 21 wherein the antibody or fragment thereof is a chimera.
- 10 23. The method of Claim 22 wherein the chimera is the variable region of a murine antibody and the constant region of a human source.
24. The method of Claim 23 wherein the variable region is from a monoclonal antibody produced by the 128.1 hybridoma.
- 15 25. The method of Claim 24 wherein the chimera is said monoclonal antibody variable region and IgG<sub>3</sub> hinge region.
- 20 26. The method of Claim 21 wherein said neuropharmaceutical agent is conjugated to the fusion protein via a cleavable link.
27. The method of Claim 26 wherein the cleavable link is formed using disulfide, cis-aconitic acid, cis-carboxylic alkadiene, cis-carboxylic alkatriene or poly-maleic anhydride.
- 25 28. The method of Claim 19 wherein said neuropharmaceutical agent is conjugated to the fusion protein via a noncleavable link.

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29. The method of Claim 28 wherein the noncleavable link is an amide bond, a bond between a sulphydryl group and a maleimide derivative, a bond between a thiol and an amino group or a bond between an aldehyde and an amino group.

5

30. A delivery system for delivering a neuropharmaceutical agent across the blood brain barrier comprising a fusion protein that includes a brain capillary endothelial cell receptor ligand and said neuropharmaceutical agent, whereby the delivery system transports the neuropharmaceutical agent across the blood brain barrier in vivo.

10

31. The delivery system of Claim 30 wherein the neuropharmaceutical agent is selected from the group consisting of a growth factor, superoxide dismutase, CD4, a lymphokine, a lymphokine antagonist, a cytokine, a cytokine antagonist, dopamine decarboxylase and tricosanthin.

15

32. The delivery system of Claim 31 wherein the neuropharmaceutical agent is a growth factor selected from the group consisting of nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), glial cell-line derived neurotrophic factor (GDNF), neurotrophin 3 (NT-3), neurotrophin 4 (NT-4), neurotrophin 5 (NT-5) and fibroblast growth factor (FGF).

20

33. The delivery system of Claim 32 wherein the neuropharmaceutical agent is NGF.

25

34. The delivery system of Claim 32 wherein the neuropharmaceutical agent is CNTF.

30

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- 5        35. The delivery system of Claim 30 wherein the ligand is selected from the group consisting of transferrin, insulin-like growth factor 1 (IGF 1), insulin-like growth factor 2 (IGF 2), insulin, an antibody to the transferrin receptor, an antibody to insulin-like growth factor 1 (IGF 1) receptor, an antibody to insulin-like growth factor 2 (IGF 2) receptor and an antibody to insulin receptor.
- 10        36. The delivery system of Claim 30 wherein the ligand and the neuropharmaceutical agent are linked through an intermediate peptide or polypeptide.
- 15        37. The delivery system of Claim 36 wherein the intermediate polypeptide is an amino acid sequence from an antibody chain.
- 20        38. The delivery system of Claim 37 wherein the intermediate polypeptide is an IgG<sub>3</sub> hinge region.
- 25        39. The delivery system of Claim 38 wherein the ligand is transferrin and the neuropharmaceutical agent is nerve growth factor (NGF).
40. The delivery system of Claim 36 wherein the ligand is an anti-transferrin receptor antibody and the neuropharmaceutical agent is nerve growth factor (NGF).
41. The delivery system of Claim 40 wherein the anti-transferrin receptor antibody is a chimeric antibody.
42. The delivery system of Claim 38 wherein the ligand is transferrin and the neuropharmaceutical agent is ciliary neurotrophic factor (CNTF).

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43. The delivery system of Claim 36 wherein the intermediate peptide is five glycines (gly)<sub>5</sub>.
- 5 44. The delivery system of Claim 43 wherein the ligand is transferrin and the neuropharmaceutical agent is nerve growth factor (NGF).
45. The delivery system of Claim 43 wherein the ligand is transferrin and the neuropharmaceutical agent is ciliary neurotrophic factor (CNTF).
- 10 46. The delivery system of Claim 36 wherein the intermediate peptide is leucine-glutamic acid (leu-glu).
47. The delivery system of Claim 46 wherein the ligand is transferrin and the neuropharmaceutical agent is nerve growth factor (NGF).
- 15 48. A delivery system for delivering a neuropharmaceutical agent across the blood brain barrier comprising a fusion protein linked to a neuropharmaceutical agent, wherein the fusion protein includes a brain capillary endothelial cell receptor ligand and an antibody or fragment thereof that is reactive with a brain capillary endothelial cell receptor, whereby the delivery system transports the neuropharmaceutical agent across the blood brain barrier when administered in vivo.
- 20 49. The delivery system of Claim 48 wherein the ligand and the antibody or fragment thereof are reactive with the transferrin receptor and the ligand is transferrin.

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50. The delivery system of Claim 49 wherein the antibody or fragment thereof is a chimera of the variable region of a murine antibody and the constant region of a human source.
- 5 51. The delivery system of Claim 50 wherein the variable region is from a monoclonal antibody produced by the 128.1 hybridoma and the constant region is an IgG<sub>3</sub> hinge region.
- 10 52. The delivery system of Claim 48 wherein said neuropharmaceutical agent is conjugated to the fusion protein via a cleavable link.
53. The delivery system of Claim 48 wherein said neuropharmaceutical agent is conjugated to the fusion protein via a noncleavable link.
- 15 54. A fusion protein comprising a brain capillary endothelial cell receptor ligand and a neuropharmaceutical agent.
55. The fusion protein of Claim 54 wherein the neuropharmaceutical agent is selected from the group 20 consisting of a growth factor, superoxide dismutase, CD4, a lymphokine, a lymphokine antagonist, a cytokine, a cytokine antagonist, dopamine decarboxylase and tricosanthin.
- 25 56. The fusion protein of Claim 55 wherein the neuropharmaceutical agent is a growth factor selected from the group consisting of nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), glial cell-line derived neurotrophic factor (GDNF), neurotrophin 3

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(NT-3), neurotrophin 4 (NT-4), neurotrophin 5 (NT-5) and fibroblast growth factor (FGF).

57. The fusion protein of Claim 56 wherein the neuropharmaceutical agent is NGF.

5 58. The fusion protein of Claim 56 wherein the neuropharmaceutical agent is CNTF.

10 59. The fusion protein of Claim 54 wherein the ligand is selected from the group consisting of transferrin, insulin-like growth factor 1 (IGF 1), insulin-like growth factor 2 (IGF 2), insulin, an antibody to the transferrin receptor, an antibody to insulin-like growth factor 1 (IGF 1) receptor, an antibody to insulin-like growth factor 2 (IGF 2) receptor and an antibody to insulin receptor.

15 60. The fusion protein of Claim 54 wherein the ligand and the neuropharmaceutical agent are linked through an intermediate peptide or polypeptide.

20 61. The fusion protein of Claim 60 wherein the intermediate polypeptide is an amino acid sequence from an antibody chain.

62. The fusion protein of Claim 61 wherein the intermediate polypeptide is an IgG<sub>3</sub> hinge region.

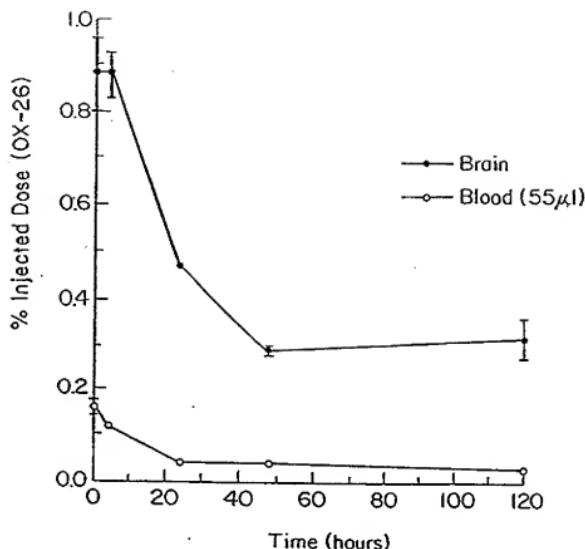
25 63. The fusion protein of Claim 62 wherein the ligand is transferrin and the neuropharmaceutical agent is nerve growth factor (NGF).

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64. The fusion protein of Claim 60 wherein the ligand is an anti-transferrin receptor antibody and the neuropharmaceutical agent is nerve growth factor (NGF).
- 5 65. The fusion protein of Claim 64 wherein the anti-transferrin receptor antibody is a chimeric antibody.
66. The fusion protein of Claim 62 wherein the ligand is transferrin and the neuropharmaceutical agent is ciliary neurotrophic factor (CNTF).
- 10 67. The fusion protein of Claim 60 wherein the intermediate peptide is five glycines (gly)<sub>5</sub>.
68. The fusion protein of Claim 67 wherein the ligand is transferrin and the neuropharmaceutical agent is nerve growth factor (NGF).
- 15 69. The fusion protein of Claim 67 wherein the ligand is transferrin and the neuropharmaceutical agent is ciliary neurotrophic factor (CNTF).
70. The fusion protein of Claim 60 wherein the intermediate peptide is leucine-glutamic acid (leu-gly).
- 20 71. The fusion protein of Claim 70 wherein the ligand is transferrin and the neuropharmaceutical agent is nerve growth factor (NGF).
72. Use of a fusion protein for the manufacture of a medicament for delivering a neuropharmaceutical agent across the blood brain barrier to the brain of a host, wherein said fusion protein includes a brain capillary

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endothelial cell receptor ligand and said  
neuropharmaceutical agent, and whereby the delivery  
occurs under conditions wherein binding of said ligand  
to its complementary brain endothelial cell receptor  
occurs and said neuropharmaceutical agent is  
transferred across the blood brain barrier in a  
pharmaceutically active form and in a therapeutically  
effective amount.

**FIG. 1**

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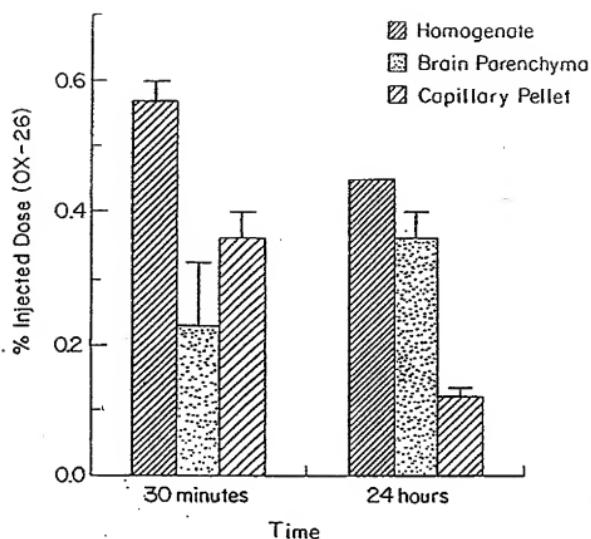


FIG. 2

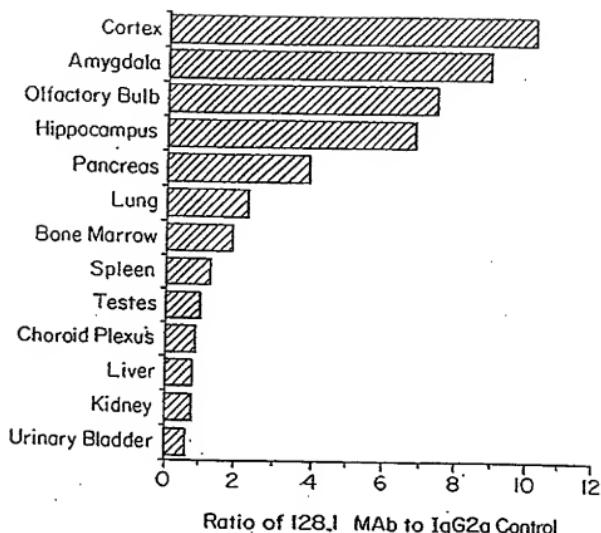


FIG. 3

SUBSTITUTE SHEET (RULE 26)

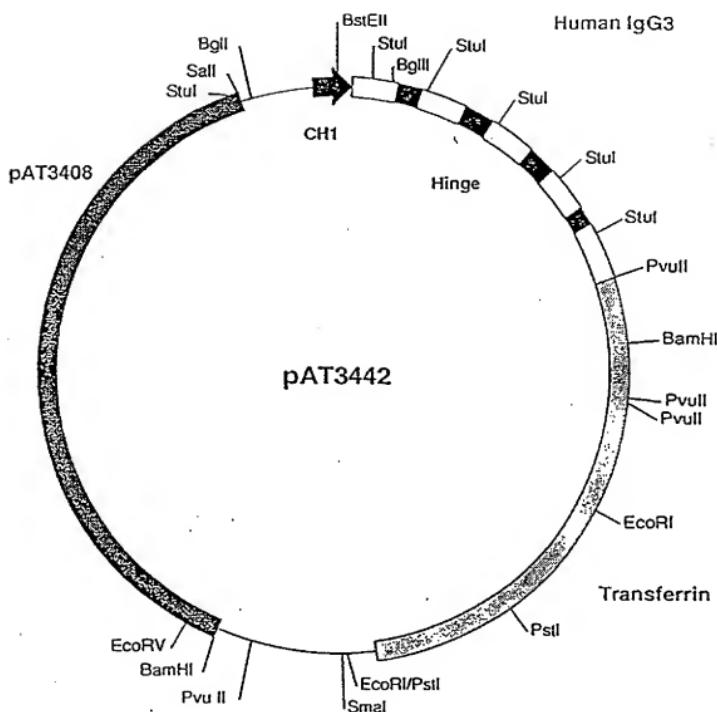


FIG. 4

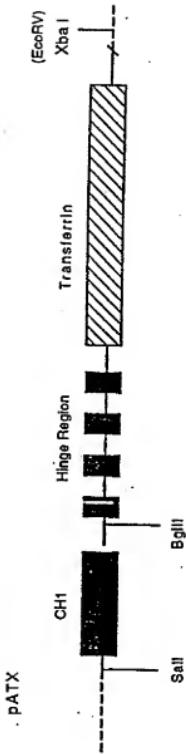


FIG. 5A

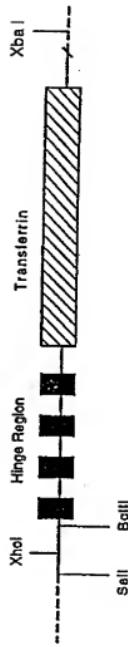


FIG. 5B

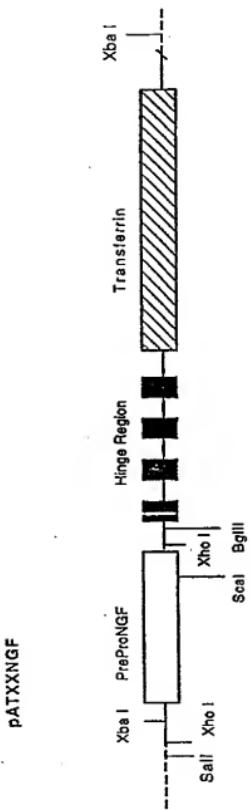


FIG. 5C

**SUBSTITUTE SHEET (RULE 26)**

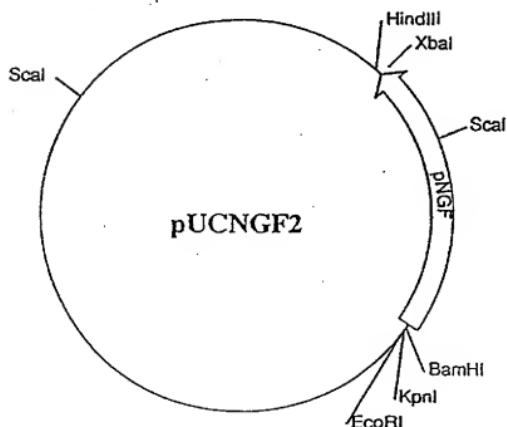
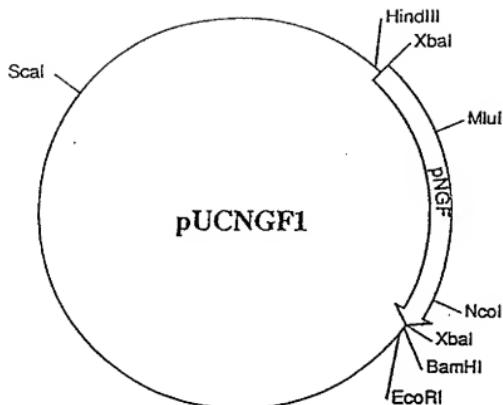


FIG. 6  
SUBSTITUTE SHEET (RULE 26)

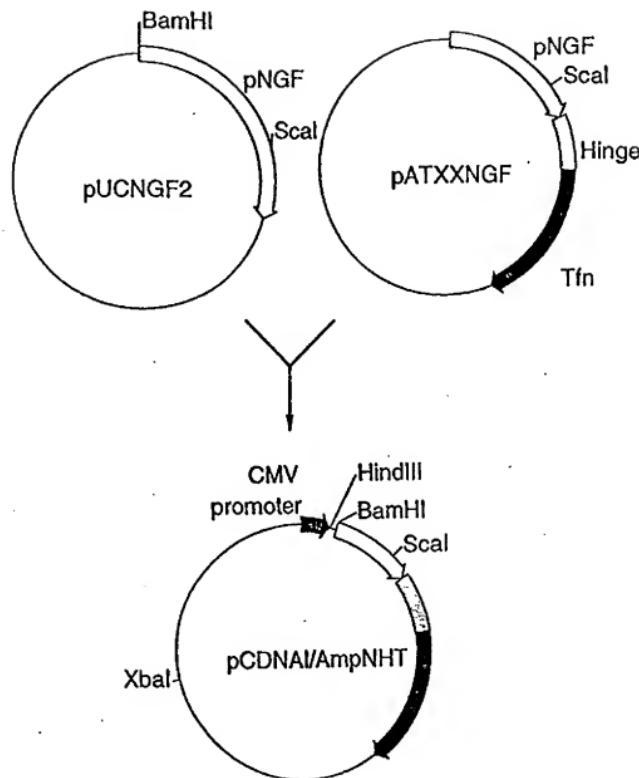


FIG. 7

SUBSTITUTE SHEET (RULE 26)

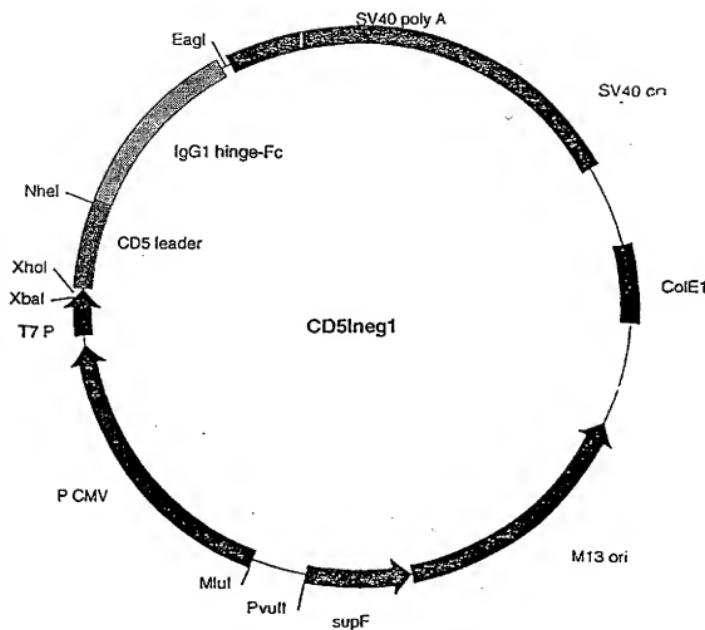


FIG. 8

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Xba I

CTCGAGATCC ATTGTGCTCT AAAGGAGATA CCCGGCCAGA CACCTCACCC TGCGGTGCC  
AGCTGCCAG GCTGAGGAA GAGAAGGCCA GAAACC

CDS Leader

ATGC CCATGGGTC TCTGCAACCG CTGGCCACCT TGTACCTGCT  
GGGGATGCTG GTCGCTTCCG TGCTAGCGGA TCCCGAG

GGT GAGTACTAAG CTTAGCGCT CTCGCTGGA CGCATCCCCG CTATGCAGCC  
CCAGTCAGG GCAGCAAGGC AGGGCCCGTC TGCTCTCTCA CCCGGAGCCT CTGGCCGCC  
CACTCATGCT CAGGGAGAGG GTCTCTGTC TTTTCCCCAG GCTCTGGGCA GGCACAGGCT  
AGGTGCCCCCT AACCCAGGCT CTGCAACAAAGGGGCAAGGT GCTGGGCTCA GACCTGCCAA  
GAGGACATATC CGGGAGGACC CTGCCCCCTGA CCTAAGGCCA CCCCAAGGC CAAACCTCTCC  
ACTCCCTAG CTGGACACC TCTCTCTCA CCAGATCCA GTAACTCCCA ATCTCTCTCA  
TGCA

IgG1 Exon 1

GAGCCC AAATCTTGTG ACAAAACTCA CACATGCCA CCGTGCCAG  
GTAAGCCAGC CCA

GGCCTCG COCTCCAGCT CAGGGGGGA CAGGTGCCCT AGAGTAGGCT GCATCCAGGG  
ACAGGCCCCA GCGGGGTGCT GACACGTCCA CCTOCATCTC TTCCCTCA

IgG1 Exon 2

GCA CCTGAACTCC TGGGGGGACC GTCACTTC CTCTTCCCC  
CAAAACCCAA GGACACCCCTC ATGATCTCCC GGACCCCTGA  
GGTCACATGC GTGGTGGTGG ACGTGAGCCA CGAAGACCC  
GAGGTCAGT TCAACTGGTA CGTGGACGGC GTGGAGGTGC  
ATAATGCCAA GACAAAGCCG CGGGAGGAGC AGTACAAACAG  
CACGTACCGG GTGGTCAGGC TCCTCACCGT CCTGACCA  
GACTGGCTGA ATGGCAAGGA GTACAAGTGC AAGGTCTCCA  
ACAAAGCCCT CCCAGCCCCC ATCGAGAAAA CCATCTCAA  
AGCCAAA

GGTGGGACCCGTG GGTTGGAGGG GGCACATGGG CAGAGGCCCCG CTGGGCCAC  
CTCTGCCCT GAGAGTGACC CTGTACCA CCTCTGTCT ACA

IgG3 Exon 3

GGGCAGC CCAGAGAACCC ACAGGTGTAC ACCCTGCC  
CATCCCCGGGA TGAGCTGACC AAGAACCAAGG TCAGCCTGAC  
CTGCTGGTC AAAGGCTTCT ATCCCAGCGA CATGCCGTG  
GAGTGGGAGA

## FIG. 9A

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GCAATGGGCA GCCGGAGAAC AACTACAAGA CCACGGCTCC  
CGTGCTGGAC TCCGACGGCT CCTTCTTCCT CTACAGCAAG  
CTCACCGTGG ACAAGAGCAG GTGGCAGCAG GGGAACGTCT  
TCTCATGCTC CGTGATGCAT GAGGCTCTGC ACAACCACTA  
CACGCAGAAG AGCCTCTCCC TGTCTCCGGG TAAA  
TGAGTG CGACGGCCG  
Eag I

**FIG. 9B**

SUBSTITUTE SHEET (RULE 26)

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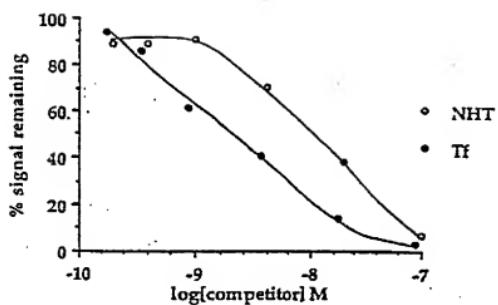


FIG. 10

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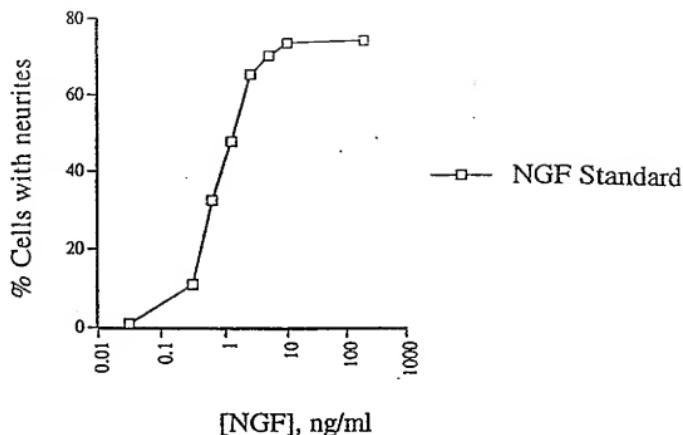
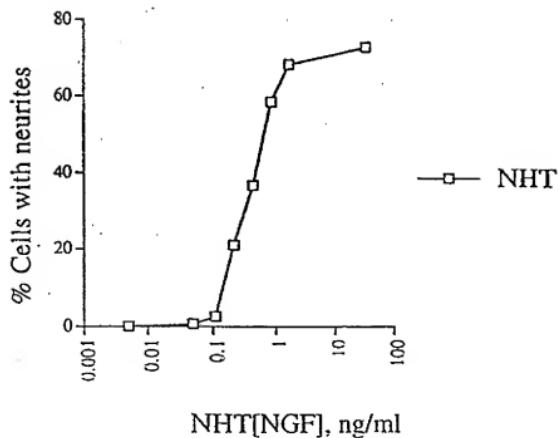


FIG. 11A

FIG. 11B  
SUBSTITUTE SHEET (RULE 26)

D1 (d1)

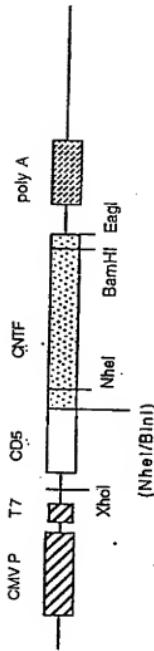


FIG. 12A

C4 (C\*)

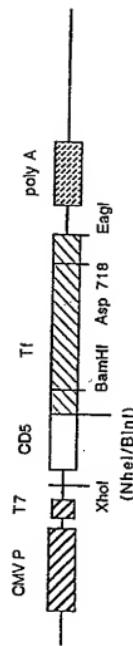


FIG. 12B

C4-NHT

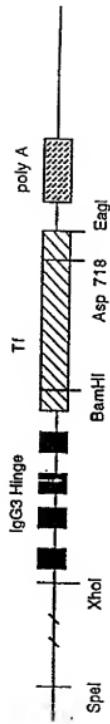


FIG. 12C

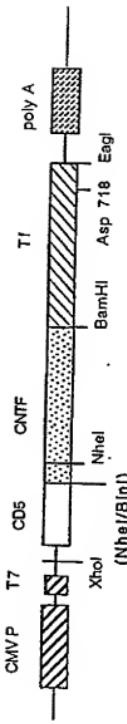


FIG. 12D

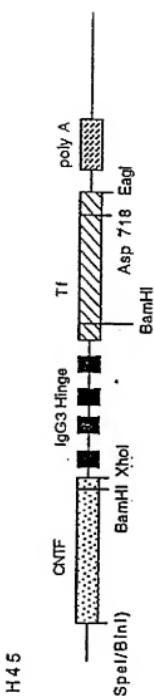


FIG. 12E



FIG. 12F

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 94/08000A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K47/48 C07K14/65

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 22332 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 23 December 1992 see page 18, line 31 - line 33; claim 8 ----- EP,A,0 466 222 (DOWELANCO) 15 January 1992 ----- see page 16, line 25 - line 47; claims 1,3,10 ----- -----	1
X		1-3,6-8, 19-22, 26-31, 35-37, 48-50, 52-55, 59-61,72

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*B\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date but not in conflict with the application but cited to understand the principle or教义 underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1 Date of the actual completion of the international search  21 November 1994	Date of mailing of the international search report  D 8. 12. 94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentsta 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax (+31-70) 340-2016	Authorized officer  Berte, M

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 94/08000

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	WO,A,94 02178 (THE GOVERNMENT OF THE UNITED STATES OF AMERICA) 3 February 1994 see page 1, paragraph 2; claims 1,18 -----	1-3,6, 19,30, 35,48
A	WO,A,93 10819 (ALKERMES) 10 June 1993 see page 7, paragraph 2 -paragraph 3 see page 8 see page 10, paragraph 3 see page 15, paragraph 3 - page 17, paragraph 2; examples 11,13,15 -----	1-53
P, X	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS), vol.269, no.7, 18 February 1994, BALTIMORE, MD US pages 4979 - 4985 SEUNG-UON SHIN ET AL. 'FUNCTIONAL PROPERTIES OF ANTIBODY INSULIN-LIKE GROWTH FACTOR FUSION PROTEINS. see results see page 4980 -----	1,2,7-9
X	WO,A,91 14438 (THE TRUSTEES OF COLUMBIA UNIVERSITY) 3 October 1991 ----- see claims 14-18,27,31-33,38,48,49- -----	1-4, 6-10, 13-15, 18-23, 26-33, 35-41, 44, 48-50, 52-57, 59-65, 68,71,72
X	EP,A,0 196 056 (CHIRON CORPORATION) 1 October 1986 see abstract see page 30; claims 1,20 -----	1,2,26, 36
P, X	EP,A,0 599 303 (TAKEDA CHEMICAL INDUSTRIES) 1 June 1994 see page 3, line 44 - page 4, line 7 see page 6, line 38 - line 42 see page 4, line 10 - line 18 -----	1-6

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 94/08000

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: 1-3, 19, 30, 48, 54, 72 because they relate to subject matter not required to be searched by this Authority, namely:  
**FOR FURTHER INFORMATION SEE ANNEX**
2.  Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

## Remark on Protest

The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

CONTINUATION OF BOX I.2

In view of the large number of compounds defined by the definition of products by means of their biological, chemical and or pharmacological properties, the search has to be restricted for economic reasons.

The search was limited to the compounds for which pharmacological data was given and/or the compounds mentioned in the claims or examples.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.  
PCT/US 94/08000

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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